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## GENETICALLY MODIFIED PLANTS WITH ENHANCED RESISTANCE TO FUNGAL DISEASES AND A METHOD OF PRODUCTION THEREOF

This application is entitled to and claims priority benefit to U.S. provisional  
5 application Serial No. 60/331,749, filed November 20, 2001, which is  
incorporated herein by reference in its entirety.

### 1. FIELD OF THE INVENTION

The present invention relates to genetically modified plants, and in particular  
10 genetically modified potato plants. The genetically modified plants have an  
enhanced resistance to pathogens following transformation of the plant with a  
vector comprising a chitinase gene with two or more chitin-binding domains and  
a  $\beta$ -1,3-glucanase gene, and in particular, a *Brassica* chitinase gene with two  
chitin-binding domains and a *Hevea*  $\beta$ -1,3-glucanase gene. The invention further  
15 relates to a recombinant vector that transforms potato plants so as to confer upon  
the potato plants immunity against pathogens. The vector comprises a chitinase  
gene having two or more chitin-binding domains; and a  $\beta$ -1,3-glucanase gene.  
The invention further relates to a method of producing an enhanced pathogen  
resistance plant by expressing a chitinase gene and a  $\beta$ -1,3-glucanase gene.

### 2. BACKGROUND OF THE INVENTION

Plants play a critical role as nutrients for animals, including humans, and for the  
production of substances useful as pharmaceuticals, cosmetics and the like.  
The steady growth in the world's population results in increasing needs for plant  
25 crops. This increased need must be satisfied with reduced soil resources  
available to agriculture. Increased crop yield can be provided with existing soil  
resources by engineering plant species that grow better and that are more  
resistant to plant pathogens.

Plants are subjected to threats by numerous pathogens, *e.g.*, fungi, bacteria,  
30 viruses, insects and nematodes. A small fraction of pathogens succeed in

invading plant tissue and thereby cause disease. In particular, potato is a major food crop that is highly susceptible to fungal infection. One strategy to combat fungal disease include the use of chemical fungicides. However, this involves high expense and environmental cost. Previous attempts to increase pathogen resistance in plants include the expression of the tomato Mi-1.2 nematode resistance gene in a nematode-susceptible tomato line. The resulting transgenic tomato plants showed resistance to the root knot nematodes *M. javanica* strain VW4 and *M. incognita* strain VW6 in most of the transgenic plants but not against *M. javanica* strain VW5, thus resembling the specificity of the Mi gene in wild-type plants (Milligan *et al.*, 1998, The Plant Cell 10:1307-1319).

In another study, plant resistance was induced by activating an inactive transgene encoding the Cf-9 resistance gene product through excision of a transposable element from that gene in a plant that expressed Avr9, a Cf-9 elicitor (WO 95/31564). Also, the plant Prf resistance gene was overexpressed in tomato plants, leading to enhanced resistance to *P. s. pathovar tomato* strain DC3000, *X. c. pv. vesicatoria* strain 56, *R. solanacearum* strain 82 bacterial pathogens and TMV viral pathogen (Oldroyd *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95:10300-10305).

In another attempt to increase plant resistance, glucose oxidase was expressed in potato plants to generate  $H_2O_2$ , a reagent produced during plant defense responses, through glucose oxidation.  $H_2O_2$  elevation in transgenic potato plants was shown to increase resistance to *E. carotovora* subspecies *carotovora* and *P. infestans* (Wu *et al.*, 1995, The Plant Cell 7:1357-1368). Transgenic rice plants were generated expressing the potato proteinase inhibitor II gene, rendering the plants more resistant to pink stem borer larvae of *Sesamia inferens* (Duan *et al.*, 1996, Nature Biotechnology 14:494-498). Also, resistance to *G. pallida* was enhanced in transgenic potato plants expressing cowpea trypsin inhibitor (U.S. Patent No. 5,494,813). The expression of a protein that disrupts the feeding structure of plant nematode pathogens is suggested in U.S. Patent No. 5,866,777 and the expression of lytic proteins in apple tree plants is discussed in

U.S. Patent No. 5,824,861.

Pathogenesis-related (PR) proteins including chitinases and  $\beta$ -1,3-glucanases are produced by plants in response to infection by pathogens. Chitinase hydrolyzes chitin, a major component of fungal cell walls while  $\beta$ -1,3-glucanase hydrolyzes the glucan component (Boller *et al.*, 1985, In: J.L. Key *et al.* ed., Cellular and Molecular Biology of Plant Stress, Alan R. Liss, New York, pp. 247-262; Boller, 1992, In: S.J. Gurr *et al.* (ed), Molecular Plant Pathology Volume II, A Practical Approach, IRL press, Oxford, pp. 23-30). Schlumbaum *et al.* (1986, Nature 324:365-367) have demonstrated that purified plant chitinases demonstrate antifungal activity *in vitro* and the expression of chitinases with one chitin-binding domain in transgenic plants enhances their resistance against fungal pathogens (Broglie *et al.*, 1991, Science 254:1194-1197; Lin *et al.*, 1995, Bio/Technology 13:686-691). In a resistant cultivar of *Brassica napus*, the mRNA of ChB4, a chitinase with one chitin binding protein, was induced by *Phoma lingam* infection and was detected within a day on northern blot analysis (Rasmussen *et al.*, 1992, Plant Mol. Biol. 20:277-287). Also, chitinases act synergistically with  $\beta$ -1,3-glucanases in inhibiting fungal growth (Mauch *et al.*, 1988, Plant Physiol. 88:936-942; Zhu *et al.*, 1994, Bio/Technology 12:807-812; Jach *et al.*, 1995, Plant J. 8:97-109)

There remains a need for plants, such as potato plants, with enhanced resistance to fungus. In particular, potato plants with enhanced resistance to soil-borne fungus.

Citation of a reference in this or in any section of the specification shall not be construed as an admission that such reference is prior art to the invention.

### 3. SUMMARY OF THE INVENTION

The present invention is based upon the observation of the present inventors that transcription and translation of *BjCH11* that encodes a chitinase with a double-chitin binding domain are induced upon fungal infection. Chitinases and  $\beta$ -glucanases are hydrolytic enzymes acting on chitin and  $\beta$ -1,3-glucan, two major

carbohydrates components of the fungal cell wall. The invention seeks to provide fungal protection in plants by transforming plants, such as crops, and specifically potato plants, with enzymes involved in the breakdown of the pathogen-related carbohydrate components and specifically fungal cell wall. In  
5 a specific embodiment, the invention provides transgenic potato plants with enhanced protection against *Rhizoctania solani*, a soil-borne fungus, by the co-expression of *Brassica juncea* chitinase with two chitin-binding domains and *Hevea*  $\beta$ -1,3-glucanase.

The present invention provides transgenic plant having therein a gene  
10 comprising a promoter, operably associated with a coding sequence for chitinase comprising two or more chitin-binding domains, and a terminator. In specific embodiments, the chitinase comprises two, three, four or five chitin-binding domains respectively. Plant cells containing a gene comprising a nucleic acid sequence encoding chitinase are also an aspect of this invention, as are other  
15 plant parts, such as for example, seed of the transformed plant containing a gene according to the invention.

In a specific embodiment, the present invention provides transgenic potato plants having therein a gene comprising a promoter, operably associated with a coding sequence for *Brassica juncea* chitinase, and a terminator. Potato plant cells  
20 containing a gene comprising a nucleic acid sequence encoding *Brassica juncea* chitinase are also an aspect of this invention, as are other plant parts, such as for example, seed of the transformed plant containing a gene according to the invention.

In a specific embodiment, the present invention further provides transgenic  
25 potato plants having therein a gene comprising a promoter, operably associated with a coding sequence for  $\beta$ -1,3-glucanase, and a terminator. Potato plant cells containing a chimeric gene comprising a nucleic acid sequence encoding  $\beta$ -1,3-glucanase are also an aspect of this invention, as are other plant parts, such as for example, seed of the transformed plant containing a gene according to the  
30 invention.

In a specific embodiment, the present invention further provides transgenic potato plants having therein a gene comprising a promoter, operably associated with a coding sequence for *Hevea*  $\beta$ -1,3-glucanase, and a terminator. Potato plant cells containing a chimeric gene comprising a nucleic acid sequence encoding *Hevea*  $\beta$ -1,3-glucanase are also an aspect of this invention, as are other plant parts, such as for example, seed of the transformed plant containing a gene according to the invention.

In a specific embodiment, the present invention further provides transgenic plant comprising a chitinase and a  $\beta$ -1,3-glucanase. In a specific embodiment, the chitinase and the  $\beta$ -1,3-glucanase are encoded in separate vectors. In another specific embodiment, the chitinase and the  $\beta$ -1,3-glucanase are encoded in the same vector. In a specific embodiment, the chitinase has two or more chitin-binding domains. In another specific embodiment, the chitinase is BjCHI1 and the  $\beta$ -1,3-glucanase is HbGLU.

In a specific embodiment, the present invention further provides transgenic plant having therein a chimeric gene comprising a first promoter, operably associated with a coding sequence for *Brassica juncea* chitinase, and a terminator, a second promoter, operably associated with a coding sequence for *Hevea*  $\beta$ -1,3-glucanase, and a second terminator. In a specific embodiment, the genetically modified plants have an enhanced resistance against fungus following the introduction, by recombinant DNA techniques, of coding sequences for *Brassica juncea* chitinase and *Hevea*  $\beta$ -1,3-glucanase. In a specific embodiment, potato (*Solanum tuberosum* L. cv. Desiree) is transformed via *Agrobacterium*-mediated transformation using pBI121-derived plant transformation plasmid that carries both *BjCHI1* and *HbGLU* cDNAs.

In another embodiment, additional gene sequences coding for plant defense proteins may be introduced into the plant in addition to the coding enzyme for chitinase and  $\beta$ -1,3-glucanase. Such gene sequences includes, but are not limited to, genes encoding ribosome-inactivating proteins, lectins and agglutinins.

In a specific embodiment, serine proteinase inhibitor may be used. In another

specific embodiment, SaPINIIa (Xu Z.F..*et al.*, 2001, Plant Molecular Biology 47:727-738) may be used.

In a specific embodiment, the present invention provides plant cells that comprise a chimeric gene. The chimeric gene comprises a nucleic acid sequence encoding a chitinase that comprises two or more chitin-binding domain and a nucleic acid sequence encoding a  $\beta$ -1,3-glucanase. In a specific embodiment, the chimeric gene comprises a nucleic acid sequence encoding a chitinase comprising four chitin-binding domains. In a specific embodiment, the plant cells are resistant to insect or bacteria or both. In a specific embodiment, the nucleic acid sequences encode *Brassica juncea* chitinase and *Hevea*  $\beta$ -1,3-glucanase.

In a specific embodiment, the present invention further provides a method of enhancing the resistance of plant to pathogens, the method comprising the steps of introducing into the plant genome a nucleic acid sequence encoding a chitinase that comprises two or more chitin-binding domain and regenerating a plant having an altered genome. In a specific embodiment, the chitinase is *Brassica juncea* chitinase under the direction of a suitable promoter and a suitable terminator.

In a specific embodiment, the present invention further provides a method of enhancing the resistance of plant to pathogens, the method comprising the steps of introducing into the plant genome a nucleic acid sequence encoding *Hevea*  $\beta$ -1,3-glucanase under the direction of a suitable promoter and a suitable terminator, and regenerating a plant having an altered genome. In a specific embodiment, the glucanase is *Hevea*  $\beta$ -1,3-glucanase under the direction of a suitable promoter and a suitable terminator.

In a specific embodiment, the present invention further provides a method of enhancing the resistance of plant to pathogens, the method comprising the steps of introducing into the plant genome a nucleic acid sequence encoding *Brassica juncea* chitinase under the direction of a suitable promoter and a suitable terminator, and *Hevea*  $\beta$ -1,3-glucanase under the direction of a suitable promoter



and a suitable terminator, and regenerating a plant having an altered genome.

#### 4. DESCRIPTION OF THE FIGURES

In order that the invention may be easily understood and readily carried into effect, reference will now be had, by way of example, to the following diagrammatic drawings in which:

Figure 1 shows the nucleotide sequence of *B. juncea* *BjCHII* and structure of *BjCHII*. A. Nucleotide sequence of *BjCHII* (SEQ ID NO:1) with deduced amino acid sequence (SEQ ID NO:2) shown under the nucleotide sequence. The chitin-binding domains are underlined. The hinge region is underlined with a dotted line. The putative polyadenylation signal is double underlined. The putative cleavage sites for the N-terminal signal peptide and the C-terminal vacuolar targeting peptide are marked (↓). The chitin-binding domains are tandem repeats that differ in two amino acids. The amino acid residues "S" at position 25 and "E" at position 30 of *BjCHII* in the first chitin-binding domain and in the second domain, amino acid residues "R" at position 76 and "A" at position 81. Positions of primers (P1 to P4) are boxed; for P2 and P4, these nucleotides correspond to their complementary sequences. *Hind*III restriction sites are shown. B. Schematic representation of *BjCHII*. The N-terminal signal peptide (black box SP) precedes two chitin-binding domains (CBD1, amino acids 22-61; CBD2, amino acids 73-112) that are separated by a spacer (gray box S). CBD2 and the chitinase catalytic domain (amino acids 146-393) are linked by a hinge region (crossed box H). Vacuolar targeting sequence, hatched box v.

Figure 2 is a comparison of *BjCHII* with other classes of plant chitinases. A. Tobacco *Chia1* (Shinshi *et al.*, 1990, Plant Mol. Biol. 14:357-368) and *Chia2* (Payne *et al.*, 1990, Proc. Natl. Acad. Sci USA 87:98-102), bean *Chia4* (Margis-Pinheiro *et al.*, 1991, Plant Mol. Biol. 17:243-253), stinging nettle *Chia5* (Lerner and Raikhel, 1992, J. Biol. Chem. 267:11085-11091) and sugar beet *Chia6* (Berglund *et al.* 1995, Plant Mol. Biol. 27:211-216). Dotted gaps are used to optimize alignment. Asterisks denote positions of identity. The putative

cleavage sites of the signal peptide and the vacuolar targeting sequence are marked ( $\downarrow$ ). The chitin-binding domains of BjCHI1 are underlined. The hinge region of BjCHI1 is underlined with a dotted line. The PPTP repeats in the spacer and hinge are marked with arrows. The synthetic peptide (YKEEIDKSDPHC), which corresponds to amino acid residues 231 to 242 of BjCHI1, that is used to raise polyclonal antibodies is boxed. The sequence NYNYG, which corresponds to amino acid residues 268 to 272 of BjCHI1, is boxed. The eight amino residues conserved in *Chia* classes are marked ( $\blacklozenge$ ). Identity (%) to BjCHI1 is shown at the end of the sequences with comparison within the chitinase catalytic domain in parenthesis. B. Schematic representation of BjCHI1, *Chia1* and UDA1 showing positions of the cysteine residues (C) involved in formation of disulfide bridges (denoted by linked lines above the bar). Black box represents signal peptide (SP); gray box, spacer (S); crossed box, hinge region (H); and hatched box, vacuolar targeting sequence (V). Figure 3 shows the result of agglutination activity assays on BjCHI1, BjCHI2, and BjCHI3. BjCHI2 and BjCHI3 are BjCHI1 derivatives that contain one and no chitin-binding domain, respectively. Agglutination assays were performed with FPLC-purified *Pichia*-expressed chitinases according to Does *et al.* (1999, *Plant Physiol.* 120:421-431). Varying amounts (0.12, 0.25, 0.5, 2, 4, 8, 16 or 24  $\mu$ g) of FPLC-purified proteins (BjCHI1, BjCHI2 and BjCHI3) from *Pichia*-expressing cultures were added to 30 microliters trypsin-treated rabbit erythrocytes in each well on a microtiter plate. Five times concentrated phosphate saline was added to a final volume of 60  $\mu$ l in each well. In the control wells (*i.e.*, "con"; last well of each row), phosphate buffered saline replaced the proteins.

Figure 4 is an analysis of *BjCHII*-related genes in the genome of *B.juncea*. A. Southern blot analysis. DNA digested with *Eco*RI (lane 1), *Hind*II (lane 2), *Hind*III (lane 3) and *Xba*I (lane 4), hybridized to  $^{32}$ P-labelled *BjCHII* cDNA, washed in 0.1 x SSC, 0.1% SDS at room temperature. Lanes 5 to 8 represent the same blot washed at 65  $^{\circ}$ C. B. PCR products with primers, P1 and P2 (lane

1) or P2 and P3 (lane 2) electrophoresed in 2% agarose gel, stained with ethidium bromide. C. Southern blot of agarose gel shown in (b) hybridized to <sup>32</sup>P-labelled *BjCHII* cDNA.

Figure 5 shows the nucleotide sequence of *Hevea*  $\beta$ -1,3-glucanase. A. Nucleotide sequence of *Hevea*  $\beta$ -1,3-glucanase (SEQ ID NO:3). B. Deduced amino acid sequence of *Hevea*  $\beta$ -1,3-glucanase (SEQ ID NO:4). C. Comparison of the deduced amino acid sequence of *Hevea* (Hb)  $\beta$ -1,3-glucanase and that of *N. plumbaginifolia* (Np) *gnl* (De Loose *et al.*, 1988, Gene 70: 12-23), Class II (CII), Class III(CIII) and Class IV (CIV)  $\beta$ -1,3-glucanase. Positions of identity are denoted by dots. The putative N-glycosylation sites in *Hevea*  $\beta$ -1,3-glucanase are marked with asterisks. The predicted N-terminal extension and C-terminal extension of *Hevea*  $\beta$ -1,3-glucanase are overlined and underlined, respectively.

Figure 6 shows genomic Southern analysis. *Hevea* genomic DNA (20 $\mu$ g) was digested with *Bam*HI (B), *Eco*RI (E), *Hind*II (H) and *Xba*I (X), separated by gel electrophoresis, blotted onto Hybond N (Amersham) membrane and hybridized with a <sup>32</sup>P-labelled *Hevea*  $\beta$ -1,3-glucanase cDNA probe.

Figure 7 shows the construction of plasmids pBj17, pHEV43 and pBj47 and pBj48. Plasmid pHEV43 was generated by cloning a 1.2 kb *Sma*I-*Hind*II fragment of full-length *HbGLU* cDNA in the *Sma*I site of plasmid pBI121, downstream from the CaMV 35S promoter. Plasmid pBj17 was generated by cloning a 1.3 kb *Sma*I fragment of full-length *BjCHII* cDNA in the *Sma*I site of pBI121. The CaMV 35S promoter drives expression of the *BjCHII* cDNA in pBj17. Subsequently the blunt-end treated 2.04 kb *Hind*III fragment from pHEV43 was cloned into the *Sna*BI of pBj17. Since this blunt-ended fragment can insert in two possible orientations, plasmids pBj47 and pBj48 were obtained, each of which contains both the *HbGLU* cDNA and the *BjCHII* cDNA on a single plasmid. In pBj47, the chitinase and the glucanase cDNAs are each transcribed from the CaMV 35S promoter in opposite directions while in pBj48, these two DNAs are transcribed in the same direction. RB, right border of T-

DNA; LB, left border of T-DNA; *nptII*, gene encoding neomycin phosphotransferase for kanamycin selection transcribed from the Nos promoter; 35S pro, CaMV 35S promoter; GUS; promoterless gene encoding  $\beta$ -glucuronidase; H, *HindIII*; E, *EcoRI*; S, *SnaBI*. The arrow above the *HbGLU* cDNA in the maps for plasmids pBj47 and pBj48 shows the direction of transcription of this cDNA from its 5'-end (5') to its 3'-end (3'). Drawings are not to scale.

Figure 8 shows BjCHII expression is induced in *B. juncea* following *R. solani* infection: (a) Northern blot analysis using  $^{32}\text{P}$ -labelled *BjCHII* cDNA of total RNA from *B. juncea* leaves harvested 0 day (lane 1), 1 day (lane 2), 2 days (lane 3), 3 days (lane 4), 4 days (lane 5), 5 days (lane 6), 6 days (lane 7), 7 days (lane 8), 8 days (lane 9) and 9 days (lane 10) after growth in *R. solani* infected soil. The same blot reprobed with a  $^{32}\text{P}$ -labelled 18S rDNA probe is shown below. The arrow denotes the 1.3 kb *BjCHII* mRNA and the arrowhead, 18S rRNA; (b) Western blot analysis using anti-BjCHII antibodies. Total protein from *B. juncea* leaves were harvested 0 day (lane 1), 1 day (lane 2), 2 days (lane 3), 3 days (lane 4), 4 days (lane 5), 5 days (lane 6), 6 days (lane 7), 7 days (lane 8), 8 days (lane 9) and 9 days (lane 10) after growth in *R. solani* infected soil. The cross-reacting 37 kDa BjCHII band is denoted with an arrow. The faint band above this band is likely a precursor protein; (c) Coomassie-stained protein gel identically loaded as in (b) to demonstrate equal amounts of protein are loaded in each well.

Figure 9 shows Northern blot analysis of  $R_0$  transgenic potato plants: A. Expression of the 1.2 kb hybridizing *HbGLU* mRNA (denoted by arrowhead) as detected with a  $^{32}\text{P}$ -labelled *HbGLU* cDNA probe in untransformed potato (lane 1) and transgenic potato lines pBj47-P<sub>10</sub> (lane 2), pBj47-P<sub>8</sub> (lane 3), pBj47-P<sub>7</sub> (lane 4) and pHEV-P<sub>14</sub> (lane 5); B. Expression of the 1.3 kb *BjCHII* hybridizing mRNA (denoted by arrow) as detected with a  $^{32}\text{P}$ -labelled *BjCHII* cDNA probe in untransformed potato (lane 1) and transgenic potato lines pBj47-P<sub>10</sub> (lane 2), pBj47-P<sub>8</sub> (lane 3), pBj47-P<sub>7</sub> (lane 4), pBI121 transformed potato (lane 5) and

pBj17-P<sub>6</sub> (lane 6).

- Figure 10 shows Southern blot analysis of R<sub>0</sub> transgenic potato plants: A. Using a <sup>32</sup>P-labelled *HbGLU* cDNA probe and *Eco*RI-digested DNA from untransformed potato (lane 1), pBI121 transformed potato (lane 2) and transgenic potato lines pHEV43-P<sub>14</sub> (lane 3), pBj47-P<sub>7</sub> (lane 4), pBj47-P<sub>8</sub> (lane 5) and pBj47-P<sub>10</sub> (lane 6). Arrowhead denotes 1.2 kb *Eco*RI-hybridizing band; B. Using a <sup>32</sup>P-labelled *BjCH11* cDNA probe and *Hind*III-digested DNA from untransformed potato (lane 1), pBI121 transformed potato (lane 2) and transgenic potato lines pBj47-P<sub>7</sub> (lane 3), pBj47-P<sub>8</sub> (lane 4), pBj47-P<sub>10</sub> (lane 5) and pBj17-P<sub>6</sub> (lane 6). Arrow denotes 0.9 kb *Hind*III-hybridizing band; C. Using a <sup>32</sup>P-labelled *BjCH11* cDNA probe and *Eco*RI-digested DNA from untransformed potato (lane 1), pBI121 transformed potato (lane 2) and transgenic potato lines pBj47-P<sub>10</sub> (lane 3), pBj47-P<sub>8</sub> (lane 4) and pBj47-P<sub>7</sub> (lane 5).
- Figure 11 shows Western blot analysis of R<sub>0</sub> transgenic potato plants: A. Western blot analysis using anti-HbGLU antibodies on crude protein from transgenic potato lines pBj47-P<sub>7</sub> (lane 1), pBj47-P<sub>8</sub> (lane 2), pBj47-P<sub>10</sub> (lane 3), pHEV43-P<sub>14</sub> (lane 4), pBI121 transformed potato (lane 5) and untransformed potato (lane 6). Band corresponding to HbGLU (35 kDa) is denoted by an arrowhead; B. Western blot analysis using anti-BjCH11 antibodies on crude protein from transgenic potato lines pBj47-P<sub>7</sub> (lane 1), pBj47-P<sub>8</sub> (lane 2), pBj47-P<sub>10</sub> (lane 3), pHEV43-P<sub>14</sub> (lane 4), pBI121 transformed potato (lane 5), untransformed potato (lane 6) and transgenic potato line pBj17-P<sub>6</sub> (lane 7). Band corresponding to BjCH11 (52 kDa) is denoted by an arrow.
- Figure 12 shows glucanase and chitinase assays on transgenic potato lines: A. Glucanase assays measured in optical density at 500 nm using crude protein from pBI121-transformant and transgenic R<sub>0</sub> lines transformed with pBj47; B. Chitinase assays measured in optical density at 550 nm using crude protein pBI121-transformant and transgenic R<sub>0</sub> lines transformed with pBj47. Error bars represent standard deviations of three separate experiments.

Figure 13 shows *In vitro* hyphal inhibition assay using *T. viride*: A. Fungal growth 16 h after addition of 50 µg protein extract from each potato line in well. Well 1: transgenic potato line pBj47-P<sub>10</sub> co-expressing BjCHI1 and HbGLU; well 2: buffer-only control; well 3: wild-type potato Desiree; well 4: potato pBI121 transformant; well 5: transgenic potato line pBj17-P<sub>6</sub> expressing BjCHI1 and well 6: transgenic potato line pHEV43-P<sub>14</sub> expressing HbGLU; B. Fungal growth of plate shown in Fig. 13A, 24 h after addition of 50 µg protein extract. Bar at lower left represents 0.9 cm.

Figure 14 shows *In vivo* fungal bioassays using *R. solani*. Potato plants were transferred to soil preinoculated with *R. solani*. The plants were examined and photographed after two weeks following transplantation: A. Transgenic potato line pBj47-P<sub>10</sub> co-expressing BjCHI1 and HbGLU; B. Transgenic potato line pBj17-P<sub>6</sub> expressing BjCHI1; C. Transgenic potato line pHEV43-P<sub>14</sub> expressing HbGLU; and D. Untransformed potato.

## 5. DETAILED DESCRIPTION OF THE INVENTION

Pathogen resistance is an important property in plants and a useful tool for the protection of plants, especially crop plants. The term “plant”, as used herein, includes whole plants, plant parts, individual plant cells, groups of plants cells (e.g., cultured plant cells) and progeny thereof. The term “enhance” when used to describe an increase of resistance of a plant to a pathogen, as used herein, includes the increase of the resistance of a plant that may have no resistance, or some resistance or substantial resistance to the pathogen to effecting the increase in resistance.

Plant pathogens include, but are not limited to, bacteria, viruses, fungi, nematodes and insects. A pathogen may infect a plant and cause severe damage to that plant, including death. Upon infection, a plant may initiate a protective reaction to the pathogen, e.g., a hypersensitive response, depending on whether the plant can recognize the pathogen.

Pathogens of the various classes may change, for example, through mutagenesis.

Also, new pathogens may arise that were not previously encountered by a plant species. For example, when a plant (*e.g.*, a crop, a fruit, a vegetable, etc.) is introduced into a continent (for example, through importation), a plant species is likely exposed to pathogens it has not encountered before.

## 5 **5.1 CLONING OF A POLYNUCLEOTIDE ENCODING *BRASSICA JUNCEA* CHITINASE AND *HEVEA* $\beta$ -1,3-GLUCANASE GENES**

### **5.1.1. Isolation of a *B. juncea* cDNA encoding a chitinase with two chitin-binding domains**

When an amplified *B. juncea* cDNA library was screened for chitinase clones using a  $^{32}\text{P}$ -labeled *Arabidopsis* basic chitinase probe (Samac *et al.*, 1990, Plant Physiol 93:907-914) prepared by PCR, five putative positives were obtained. Nucleotide sequence analysis showed that three clones had a 1.3 kb cDNA, designated *BjCHII*, while the other two were incomplete-length cDNAs. *BjCHII* cDNA consists of 3 bp 5'-untranslated region, 1200 bp translated region, 86 bp 3'-untranslated region and a poly (A)+ tail (Figure 1a). An open reading frame of 400 amino acids encodes a protein of predicted  $M_r$  42,774 (isoelectric point 4.7) with homology to plant *Chia1* chitinases. Interestingly, this cDNA contains two 120 bp-repeats (nucleotides 67-186 and 220-339, Figure 1a), which encode two chitin-binding domains linked by a 11-amino acid spacer; they differ in seven nucleotides resulting in two amino acid changes. A thirty-three amino acid hinge links the second chitin-binding domain to the chitinase catalytic domain (Figure 1b). All other previously characterized chitinases contain only one chitin-binding domain. The chitin-binding domain is essential for chitin binding (Iseli *et al.*, 1993, Plant Physiol. 103:221-226) and at least two such domains are required for cell agglutination (Peumans *et al.*, 1995, Plant Physiol. 109:347-352). Occurrences of the chitin-binding domain are not limited to chitinases. This domain is also present in plant lectins, *e.g.* hevein, a 4.7 kDa protein from rubber latex (Van Parijs *et al.*, 1991, Planta 183:258-264) has one chitin-binding domain. *BjCHI1* is a unique chitinase with two chitin-binding

domains and the present inventions have shown that these two chitin binding domains confer agglutination properties using *Pichia*-expressed BjCHI1 (see Section 5.1.2, *infra*). A chitinase with agglutination properties has not been previously reported.

### 5 5.1.2. Comparison of BjCHI1 with other chitinases

Figure 2A shows the alignment of BjCHI1 with representatives of different chitinase classes: *Chia1* from *Nicotiana tabacum* (Shinshi *et al.*, 1990, Plant Mol Biol 14:357-368), *Chia2* from *N. tabacum* (Payne *et al.*, 1990, Proc Natl Acad Sci USA 87:98-102), *Chia4* from *P. vulgaris* (Margis-Pinheiro *et al.*, 1991, Plant Mol Biol 17:243-253), *Chia5* from *U. dioica* (Lerner *et al.*, 1992, J Biol Chem 267:11085-11091) and *Chia6* from *B. vulgaris* (Berglund *et al.*, 1995, Plant Mol Biol 27:211-216). The representatives of *Chia1* and *Chia2* chitinases are those from *N. tabacum* that show highest identity to BjCHI1. Analysis of eighty-six plant chitinases has shown that eight amino acids are conserved within the *Chia* classes (Levorson *et al.*, 1997, Plant Mol Biol Repr 15:122-133). Seven of these are conserved in BjCHI1 (E-201, A-203, T-213, C-230, N-270, P-286 and G-377, Figure 2A). The eighth residue (Q) is substituted by M-264 in BjCHI1 (Figure 2A). Although BjCHI1 shares high identity to *N. tabacum Chia1* (62.0%) and *Chia2* (54.8%), it has two chitin-binding domains whereas *Chia1* has one chitin-binding domain and *Chia2* lacks this domain.

When compared with *Chia1* chitinases from other plants, BjCHI1 shares 72.7% identity to *B. napus* (Hamel *et al.*, 1993, Plant Physiol 101:1403), 70.2% identity to *A. thaliana* (Samac *et al.*, 1990, Plant Physiol 93:907-914), 60.9% identity to *P. vulgaris* (Broglie *et al.*, 1986, Proc Natl Acad Sci USA 83:6820-6824) and 51.8% identity to *Oryza sativa* (Zhu *et al.*, 1994, BioTechnology 12:807-812). BjCHI1 also contains the sequence "NYNYG" (amino acids 268 to 272, Figure 2) highly-conserved in *Chia1* chitinases (Verburg *et al.*, 1992, J Biol Chem 267:3886-3893). Investigations on the catalytic site of a *Zea mays* chitinase have shown that modification of the first Y in this sequence "NYNYG" with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide resulted in loss of activity



(Verburg *et al.*, 1992).

The presence of two chitin-binding domains would identify BjCHI1 with *Chia5*, of which only one example, *Urtica dioica* agglutinin, UDA1, is known (Levorson *et al.*, 1997). UDA1 is a 372-amino acid precursor of stinging nettle  
5 (*U. dioica*) agglutinin (Lerner and Raikhel, 1992, J Biol Chem 267:11085-11091) and shows insect antinutrient activity (Chrispeels *et al.*, 1991, Plant Cell 3:1-9). This precursor consists of a signal peptide, two chitin-binding domains and a chitinase catalytic domain. The 8.5kD mature lectin consists of only 86-amino acids with two chitin-binding domains since the chitinase domain is cleaved-off  
10 post-translationally (Lerner and Raikhel, 1992). Although BjCHI1 and UDA1 have structural similarities they share only 36.9% identity (Figure 2A). Comparison of disulfide bridges in chitinases, according to Beintema (Beintema, 1994, FEBS Letters 350:159-163), shows similarities between BjCHI1, *Chial* and UDA1 (Figure 2B); the C-terminal disulfide bridge of *Chial*, absent in  
15 UDA1, is present in BjCHI1. Unlike UDA1, the hinge and the spacer of BjCHI1 are proline-rich, with 63.6% and 54.5% proline residues, respectively. The sole member of *Chia6* chitinase (Levorson *et al.*, 1997), *B. vulgaris* Ch1, contains a hinge domain of 128 residues with 70.3% proline (Berglund *et al.*, 1995, Plant Mol Biol 27:211-216). BjCHI1 and Ch1 both contain "PPTP"  
20 repeats (Figure 2A). In contrast, Ch1 has only half a chitin-binding domain and there is only 45.2% identity between them.

The presence of the two-chitin binding domains in BjCHI1 prompted the present inventors to explore the BjCHI1's possibility of having agglutination properties. Accordingly, agglutination assays were performed with FPLC-purified *Pichia*-  
25 expressed BjCHI1 according to Does *et al.* (1999, Plant Physiol. 120:421-431). As controls, BjCHI2 and BjCHI3 containing one and no chitin-binding domain, respectively, were employed. Briefly, BjCHI1, BjCHI2 and BjCHI3 were expressed in *Pichia pastoris* using yeast expression vector pPIC9K (Invitrogen). BjCHI1, BjCHI2 and BjCHI3 were cloned in-frame to the secretory signal  
30 peptide on pPIC9K, so that the fusion proteins would be secreted in the growth

media. BjCHI1 contains both chitin-binding domains within amino acid residues 18 to 393 of the native protein (Zhao and Chye, 1999, Plant Mol. Biol. 40:1009-1018), fused to the secretory signal peptide on pPIC9K; BjCHI2 contains only one chitin-binding domain while BjCHI3 lacks both chitin-binding domains. BjCHI1, BjCHI2 and BjCHI3 DNAs were cloned into the *EcoRI* and *NotI* sites on vector pPIC9K. To this end the following primers were used in PCR: C1(forward) primer 5' CTGAATTCTCCTCC GGTGAGCAATGCG 3' (SEQ ID NO:5); C21(forward) primer 5' CTGAATTCGGGGATCTTTCTGGCATC 3' (SEQ ID NO:6); and C2(reverse) primer 5'GCGACTGCGGCCGCGTTACTACCTTCATTAAACG 3' (SEQ ID NO:7). The forward primers were designed with one *EcoRI* site and the reverse with one *NotI* site. The 1.1 kb DNA encoding BjCHI1 was amplified by PCR with C1 and C2 primers and pBj17 as template in PCR. The 0.95 kb DNA fragment encoding BjCHI2 (with one chitin-binding domain) was amplified by PCR with C1 and C2 primers and pBj28 as template. Plasmid pBj28 has been previously described in Fung *et al.* (2002, Plant Molecular Biology 50: 283-294). The 0.74 kb DNA encoding BjCHI3 (that lacks both two chitin-binding domains) was obtained by PCR using C21 and C2 primers and pBj17 as template. The PCR-amplified fragments were digested with *EcoRI* and *NotI* and cloned in the *EcoRI* and *NotI* sites of pPIC9K. The inserts in pPIC9K were analyzed by DNA sequence analysis before use in yeast transformation. The *Pichia*-expressed proteins were secreted in the growth media and precipitated using 65% ammonium sulfate according to the instructions provided by Invitrogen. Subsequently the crude extracts were purified by FPLC and the FPLC-purified proteins checked by western blot analysis with antibodies against BjCHI1 before use in agglutination assays. Since the peptide used in the preparation of polyclonal antibodies against BjCHI1 are retained within the BjCHI2 and BjCHI3 peptides, these BjCHI1 derivatives cross-reacted to these antibodies.

In agglutination assays, varying amounts (0.12, 0.25, 0.5, 2, 4, 8, 16 or 24  $\mu$ g) of

FPLC-purified proteins (BjCHI1, BjCHI2 and BjCHI3) from *Pichia*-expressing cultures were added to 30 µl trypsin-treated rabbit erythrocytes in each well on a microtiter plate (*see* Figure 3). Five times concentrated phosphate saline was added to a final volume of 60 µl in each well. In the control wells, phosphate buffered saline replaced the proteins.

The results suggest that BjCHI1, by its agglutination properties, resembles insecticidal proteins that contain at least two chitin-binding domains including wheat germ agglutinin, *Urtica dioica* agglutinin, and lectins from tomato, rice and *Datura* (Chrispeels *et al.*, 1991, *supra*). Since it has been suggested that plant lectins with two or more chitin-binding domains agglutinate cells by binding to glycoconjugates on bacterial and fungal surfaces and on the exoskeleton and intestinal lumen of herbivorous insects (Peumans *et al.*, 1995, *supra*), the anti-microbial and anti-insect effects of BjCHI1 appear promising and warrant further investigations.

### 5.1.3. Genomic organization of *BjCHII* in *B. juncea*

Southern blot analysis was used to investigate the presence of *BjCHII*-related genes in *B. juncea*. Genomic DNA digested with *EcoRI*, *HindII*, *HindIII* and *XbaI* was hybridized to *BjCHII* cDNA. Only *HindIII* cleaves within the cDNA (Figure 1A). Detection of six to nine hybridizing bands (Figure 4A, lanes 1-4) indicates related genes in heterotetraploid *B. juncea*, a hybrid of *B. nigra* and *B. campestris*; bands could be due to genes encoding chitinases or proteins with chitin-binding domain(s). Further washing of the blot at 65°C revealed fewer bands; bands in lanes 6-8 (Figure 4A), are likely *BjCHII*-specific. To investigate the occurrence of chitinases with one chitin-binding domain, PCR analysis was carried out using strategically-positioned primers. Primer P1 is adjacent to the region encoding the first chitin-binding domain while P2 is in the region encoding the chitinase domain that shows homology to other chitinases (Figure 1A, Figure 2). PCR amplification of *BjCHII* with these primers should give a 0.5 kb product, if no introns are between them; the first intron of the *Arabidopsis* chitinase gene (Samac *et al.*, 1990) is located at a corresponding

region after P2. The expected 0.5 kb product on agarose gel electrophoresis (Figure 4B) hybridized to the *BjCH11* cDNA on Southern blot analysis, together with a weaker 0.35 kb band (Figure 4C). This smaller band suggests the presence of a related chitinase with one chitin-binding domain and is weaker due to the specificity of the primers to *BjCH11*. In contrast, PCR amplification of *BjCH11* with primers P2 and P3, shows a single band of 0.35kb (Figure 4B and C, lane 2). P3, located in the region encoding the spacer (Figure 1A), is *BjCH11*-specific and was deemed suitable for measuring *BjCH11*-specific mRNA expression in RT-PCR.

#### 5.1.4. Isolation of a *Hevea* $\beta$ -1,3-glucanase gene

*N. plumbaginifolia* cDNA encoding  $\beta$ -1,3-glucanase, *gnl* cDNA (De Loose *et al.*, 1988, Gene 70:12-23) was used as a heterologous hybridization probe to isolate the corresponding cDNA clones from *Hevea*. A cDNA library prepared from *Hevea* latex was screened by *in situ* plaque hybridization at 42°C in a solution containing 30% formamide. Several putative *Hevea* cDNA clones encoding  $\beta$ -1,3-glucanase were isolated. Nucleotide sequence analysis carried out on two of the longest clones of 1.2 and 1.1kb showed that they belonged to the same class. The full-length 1.2kb cDNA consists of a 40bp 5'-untranslated region, a 1125 bp coding region, a 76 bp 3'-untranslated region and a poly(A) tail. The coding region encodes a 374 amino acid basic protein with a predicted  $M_r$  41,305.

The nucleotide sequence of *Hevea*  $\beta$ -1,3-glucanase shows 68% nucleotide sequence identity to that of the *N. plumbaginifolia* *gnl* cDNA. Comparison of the predicted amino acid sequence of *Hevea*  $\beta$ -1,3-glucanase with that of the Class I  $\beta$ -1,3-glucanase encoded by *gnl* shows 66% amino acid homology (Figure 5C). *Hevea*  $\beta$ -1,3-glucanase has 54%, 60% and 51% amino acid identity to Class II (*N. tabacum* PR-N (Linthorst *et al.*, 1990, Proc Natl Acad Sci USA 87:8756-8760)), Class III (*N. tabacum* ec321391 (Payne *et al.*, 1990, Plant Mol Biol 15:797-808) and Class IV (*N. tabacum* sp41a (Ori *et al.*, EMBO J, 1990, 9:3429-3436))  $\beta$ -1,3-glucanase, respectively (Figure 5C).

### 5.1.5 *Hevea* $\beta$ -1,3-glucanase

Class I  $\beta$ -1,3-glucanase are synthesized as preproteins and the N-terminal extension and C-terminal extension are cleaved during or after transport of the protein to the vacuole (Shinshi *et al.*, 1988, Proc Natl Acad Sci USA 85:5541-5545). The presence of an N-terminal extension (amino acid residues 1 to 36) and a C-terminal extension (amino acid residues 353 to 374) on the deduced amino acid sequence of *Hevea*  $\beta$ -1,3-glucanase further suggests that it belongs to Class I  $\beta$ -1,3-glucanase (Figure 5C). The N-terminal extension of *Hevea*  $\beta$ -1,3-glucanase consists of a region (amino acid residues 4 to 19) enriched in serine and threonine residues, followed by a hydrophobic region (amino acid residues 22 to 29) (Figure 5C). Although there is no significant amino acid sequence homology between the N-terminal of *Hevea*  $\beta$ -1,3-glucanase and *gnl*-encoded  $\beta$ -1,3-glucanase, they both consist of a hydrophobic region typical of signal peptides (Von Heijne *et al.*, 1985, J. Mol. Biol. 184:99-105) and is believed to be involved in protein targeting to the vacuole. Interestingly the N-terminal sequence of the propeptide of barley aleurain which has been shown be responsible for directing the protein to the vacuole is also rich in serine residue (Holwerda *et al.*, 1992, Plant Cell 4:307-318). Comparison of the C-terminal extension of *Hevea* with that of *N. plumbaginifolia* shows that there is some conservation in amino acid sequence and in the putative N-glycosylation site (amino acid 364 in *Hevea*  $\beta$ -1,3-glucanase). The C-terminal extension, particularly amino acid residues 365 to 370 in *Hevea*, is rich in hydrophobic amino acids. It has been suggested that a hydrophobic/acidic motif structure, rather than the specific amino acid sequence forms a sorting signal in carboxy-extension propeptides (Nakamura *et al.*, 1993, Plant Physiol 101:1-5). It has been established that the C-terminal extension and N-glycan of Class I isoforms of  $\beta$ -1,3-glucanase are removed during processing (Shinshi *et al.*, 1988).

### 5.1.6. Genomic organization of *Hevea* $\beta$ -1,3-glucanase

The laticifer-specific cDNA which the present inventors had isolated was used in

genomic Southern blot analysis to investigate the presence of a  $\beta$ -1,3-glucanase gene family in *Hevea*. Genomic DNA was obtained from young leaves following the procedure of Dellaporta *et al.*, 1983, Plant Mol Biol Rep 1: 19-21. Total genomic DNA was restricted with *Bam*HI, *Eco*RI, *Hind*III and *Xba*I, electrophoresed and blotted. Southern blot analysis using the 1.2kb *Hevea*  $\beta$ -1,3-glucanase probe showed that there were 2-4 hybridizing bands with each digest (Figure 6). These results suggest that a low-copy gene family of  $\beta$ -1,3-glucanase is present in *Hevea*.

Other chitinases and  $\beta$ -1,3-glucanases may be isolated and characterized using techniques known in the art. A cDNA or genomic DNA specific for *Brassica juncea* chitinase and *Hevea*  $\beta$ -1,3-glucanase protein or nucleic acid may be cloned and sequenced in a variety of ways, *e.g.*, dideoxy chain termination sequencing, *see, e.g.*, Sambrook *et al.*, *supra*.

The polynucleotides that may be used in the present invention include polynucleotides having the DNA sequences presented herein, and additionally include any nucleotide sequence encoding a contiguous and functional chitinase and  $\beta$ -1,3-glucanase encoding open reading frame (ORF) that hybridizes to a complement of the DNA sequences presented herein under highly stringent conditions. By way of example and not limitation, high stringency hybridization conditions can be defined as follows: The filter-bound DNA were hybridized in a solution containing 50% deionized formamide, 6xSSC, 5x Denhardt's, 1% SDS, 100  $\mu$ g/ml denatured salmon sperm DNA at 42°C overnight (about 4-16 hours), and washing in 0.1xSSC, 0.1% SDS at 65°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York) and encodes a functionally equivalent gene product.

For oligonucleotide probes, by way of example and not limitation, highly stringent conditions may refer, *e.g.*, to washing in 6XSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos).

Additionally contemplated polynucleotides that may be used in the present invention include any nucleotide sequences that hybridize under moderately stringent conditions to the complement of the DNA sequences that encodes a chitinase or  $\beta$ -1,3-glucanase. By way of example but not limitation, such  
5 moderately stringent conditions may include, *e.g.*, washing in 0.2XSSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, *supra*).

Additionally contemplated polynucleotides that may be used in the present invention include any nucleotide sequences that hybridize under low stringency conditions to the complement of the DNA sequences that encode a chitinase or  
10  $\beta$ -1,3- glucanase. By way of example and not limitation, procedures using such conditions of low stringency are described in Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792.

Moreover, a variant of chitinase and  $\beta$ -1,3-glucanase can also be used in the present invention. A variant may comprise one or more changes in the amino  
15 acid sequence of the enzyme, *e.g.*, by way of addition, substitution, or deletion of one or more amino acids, compared with the wild type enzyme. Any change should not abolish the ability of the enzyme to perform its function, though it may increase or decrease this ability depending on the nature of the changes. Preferably, the amino acid changes are conservative.

20 In various embodiments, the chitinase and  $\beta$ -1,3-glucanase, fragment, variant, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the enzyme, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence (of a different protein)). Such a chimeric gene product can be made by ligating the appropriate nucleic acid  
25 sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Preferably, the fragment, analog, and derivative of the enzyme in  
30 the fusion protein retains the ability to perform the enzyme's function.

A cDNA or genomic DNA specific for a plant chitinase and  $\beta$ -1,3-glucanase may be cloned through screening a cDNA or genomic DNA library. Such a library may be prepared, for example, from messenger RNA or genomic DNA from the plant. For general background on molecular biology techniques and  
5 on how to prepare a cDNA library and a genomic library, *see, e.g.*, Ausubel F.M. *et al., supra*; Sambrook *et al.*, 1989, *supra*; and U.S. Patent No. 5,650,148.

The library may be screened with a nucleotide fragment specific for a part of the *Brassica juncea* chitinase and *Hevea*  $\beta$ -1,3-glucanase. For example, the protein sequence of a chitinase and  $\beta$ -1,3-glucanase may be determined using techniques  
10 well known to those of skill in the art, such as via the Edman degradation technique. (*See, e.g.*, Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H. Freeman & Co., New York, pp. 34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen a cDNA library for the cDNA sequence  
15 encoding the chitinase and  $\beta$ -1,3-glucanase.

Or, for example, two stretches of protein sequences specific for the chitinase and  $\beta$ -1,3-glucanase may be determined. A set of degenerate oligonucleotides specific for each stretch is prepared and the oligonucleotides are used in a polymerase chain reaction ("PCR") amplification. Oligonucleotides are at least  
20 about 6 nucleotides long, more preferably at least about 10, more preferably at least about 15, more preferably at least about 20, more preferably at least about 30, more preferably at least about 40 nucleotides. The template in the PCR reaction would be, for example, a mixture of cDNA or genomic DNA that is known to contain or suspected to contain a DNA polynucleotide specific for the  
25 chitinase and  $\beta$ -1,3-glucanase of interest. A cDNA template may be obtained in a variety of ways, for example, by isolating a mixture of different cDNA species from a cDNA library or, for example, by reverse transcribing total mRNA from a cell or organism known to (or suspected to) express the chitinase and  $\beta$ -1,3-glucanase. For background on PCR, *see, e.g.*, Ausubel, *supra*, and PCR  
30 Protocols: A Guide to Methods and Applications, 1990, Innis, M. *et al.*, eds.



Academic Press, Inc., New York.

In order to clone a full length cDNA or genomic DNA sequence from any species or to clone variant or heterologous forms of the chitinase and  $\beta$ -1,3-glucanase, labeled DNA probes made from nucleic acid fragments corresponding to any of the polynucleotides discussed herein or made using the methods of the invention may be used to screen a cDNA library or a genomic DNA library (for example, a phage library) as described in, *e.g.*, Ausubel F.M. *et al.*, *supra*; Sambrook *et al.*, 1989, *supra*.

## 5.2 PRODUCTION OF ANTIBODIES

For the production of antibodies, various host animals may be immunized by injection with the chitinase and  $\beta$ -1,3-glucanase (*e.g.*, one corresponding to functional domain of the chitinase and  $\beta$ -1,3-glucanase), truncated chitinase and  $\beta$ -1,3-glucanase polypeptides (a chitinase or  $\beta$ -1,3-glucanase in which one or more domains have been deleted), functional equivalents of the chitinase and  $\beta$ -1,3-glucanase, mutants of the chitinase and  $\beta$ -1,3-glucanase, or short peptides (or fragments) of chitinase and  $\beta$ -1,3-glucanase. Such host animals may include but are not limited to rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Antibodies useful for the present invention include monoclonal antibodies (*see, e.g.*, Kohler *et al.*, 1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), chimeric antibodies (*see, e.g.*, Morrison *et al.*, 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger *et al.*, 1984, Nature, 312:604-608; Takeda *et al.*, 1985, Nature, 314:452-454), single chain antibodies (*see, e.g.*, U.S. Patent 4,946,778;

Bird, 1988, Science 242:423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward *et al.*, 1989, Nature 341:544-546), antibody fragments (*see, e.g.*, Huse *et al.*, 1989, Science, 246:1275-1281), anti-idiotypic antibodies or Fab fragments of such anti-idiotypes (*see, e.g.*, Greenspan & Bona, 5 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438).

### 5.3 EXPRESSION OF CHITINASE AND $\beta$ -1,3-GLUCANASE USING RECOMBINANT DNA TECHNOLOGY

Chitinase and  $\beta$ -1,3-glucanase, fragments thereof or fusion proteins thereof, are  
10 advantageously produced by recombinant DNA technology using techniques well known in the art. Such methods can be used to construct chimeric gene or expression vectors containing a chitinase or  $\beta$ -1,3-glucanase nucleotide sequence and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques,  
15 and *in vivo* genetic recombination. *See*, for example, the techniques described in Sambrook *et al.*, 1989, *supra*, and Ausubel *et al.*, 1989, *supra*. As used herein, the term chimeric gene refers to a combination of nucleic acid sequences for each part of the chimeric gene, which sequences have been engineered into relationship by recombinant DNA techniques, which sequences may also be in  
20 their separate parts endogenous or exogenous to the plant into which the chimeric gene is to be introduced.

Alternatively, RNA corresponding to all or a portion of a transcript encoded by a chitinase or  $\beta$ -1,3-glucanase nucleotide sequence may be chemically synthesized using, for example, synthesizers. *See*, for example, the techniques described in  
25 "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

Any of host-expression vector system known in the art of biotechnology may be utilized to express the chitinase or  $\beta$ -1,3-glucanase nucleotide sequence including, but not limited to, expression in bacteria, yeast, insect cells,  
30 mammalian cells, eukaryotic cells and plant cells. In these expression systems,

any selection system may be used. Such selection may comprise growth on a selective medium (*e.g.*, antibiotics, minimal media, etc.) or the use of an indicator (*e.g.*, a dye, a fluorescent reagent, etc.).

In cases where plant expression vectors are used, the expression of the chitinase  
5 and  $\beta$ -1,3-glucanase coding sequence may be driven by any of a number of regulatory elements. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson *et al.*, 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu *et al.*, 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of  
10 RUBISCO (Coruzzi *et al.*, 1984, EMBO J. 3:1671-1680; Broglie *et al.*, 1984, Science 224:838-843); or heat shock promoters, *e.g.*, soybean hsp17.5-E or hsp17.3-B (Gurley *et al.*, 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, biolistics/particle  
15 bombardment, microinjection, electroporation, *etc.* For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, New York, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9. As used herein, regulatory elements include but are not limited to  
20 inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression.

Preferably the promoter is capable of directing expression in a particular tissue of the plant and/or at particular stages of development of the plant. The promoter may be heterologous or homologous to the plant. Preferably the  
25 promoter directs expression to the endosperm of the plant seed or to the roots or tuber of the plant. A preferred promoter is the high molecular weight glutenin (HMWG) gene of wheat. Other suitable promoters will be known to the skilled man, such as the promoters of gliadin, branching enzyme, ADPG pyrophosphorylase, starch synthase and actin, for example.

## 5.4 TRANSGENIC PLANTS EXPRESSING CHITINASE AND $\beta$ -1,3-GLUCANASE

A transgenic plant with the ability to express a plant chitinase or  $\beta$ -1,3-glucanase polypeptide may be engineered by transforming a plant cell with a gene construct comprising a sequence encoding a plant chitinase and  $\beta$ -1,3-glucanase protein or polypeptide. In one embodiment, a plant promoter is operably associated with a sequence encoding the desired plant chitinase or  $\beta$ -1,3-glucanase protein or polypeptide. As used herein, the term "Operably associated" or "operably linked" refers to an association in which the regulatory regions (e.g., promoter, enhancer) and the nucleic acid sequence to be expressed are covalently joined and positioned in such a way as to permit transcription, and under the appropriate condition, translation. In a preferred embodiment of the present invention, the associated promoter is a strong and non tissue- or developmental-specific plant promoter (e.g., a promoter that strongly expresses in many or all plant tissue types). Examples of such strong, "constitutive" promoters include, but are not limited to, the CaMV 35S promoter (Odell *et al.*, 1985, Nature 313:810-812), the T-DNA mannopine synthetase promoter, and their various derivatives. In another preferred embodiment, an inducible or repressible promoter is used to express the chitinase and/or  $\beta$ -1,3-glucanase of interest in a plant, for example, a tet operator promoter as described in Weinmann *et al.*, 1994, The Plant Journal 5:559-569; or a glucocorticoid-inducible promoter as described in McNellis *et al.*, 1998, The Plant Journal 14:247-257; or an ethanol inducible promoter as described in Caddick *et al.*, 1998, Nature Biotechnology 16:177-180. See, also, Gatz, 1995, Methods In Cell Biology 50:411-424, which describes inducible and repressible gene expression systems for plants.

In one embodiment of the invention, a chitinase and/or  $\beta$ -1,3-glucanase is expressed in a plant so that the chitinase and/or  $\beta$ -1,3-glucanase polypeptide will be localized in the apoplastic space. The chitinase and/or  $\beta$ -1,3-glucanase may be directed to the apoplastic space, when expressed in a plant, by expressing the

- chitinase and/or  $\beta$ -1,3-glucanase polypeptide as a fusion protein together with a peptide that acts as a signal or transporter so that chitinase and/or  $\beta$ -1,3-glucanase is localized in the apoplastic space of the transgenic plant. A variety of signal or transporter peptides can be used, for example, the PR1b signal sequence as described in Lund *et al.*, 1992, *Plant Molecular Biology* 18:47-53; or the PR-1a, b and c signal sequences as described in Pfitzner *et al.*, 1987, *Nucleic Acids Research* 15:4449-4465. A fusion protein comprising a signal or transporter peptide and a chitinase and/or  $\beta$ -1,3-glucanase polypeptide may be constructed by linking polynucleotides specific for each component to each other (e.g., the polynucleotides are linked in frame) so that the desired fusion protein is made when the fusion polynucleotide is expressed in a transgenic plant. A skilled artisan would know how to construct a polynucleotide useful for expressing a chitinase and/or  $\beta$ -1,3-glucanase in the apoplastic space of a transgenic plant.
- 15 In another embodiment of the present invention, it may be advantageous to engineer a plant with a gene construct comprising a sequence encoding a plant chitinase and  $\beta$ -1,3-glucanase protein or polypeptide operably associated with a tissue- or developmental-specific promoter, such as, but not limited to, the CHS promoter, the PATATIN promoter, etc.
- 20 In yet another embodiment of the present invention, it may be advantageous to transform a plant with a gene construct comprising a sequence encoding a plant chitinase and  $\beta$ -1,3-glucanase protein or polypeptide operably linked to a modified or artificial promoter. Typically, such promoters, constructed by recombining structural elements of different promoters, have unique expression patterns and/or levels not found in natural promoters. See, e.g., Salina *et al.*, 1992, *Plant Cell* 4:1485-1493, for examples of artificial promoters constructed from combining cis-regulatory elements with a promoter core.
- 25 In yet an additional embodiment of the present invention, the expression of a chitinase and  $\beta$ -1,3-glucanase polynucleotide may be engineered by increasing the copy number of the gene encoding the desired protein or polypeptide using
- 30

techniques known in the art.

### **5.5 TRANSFORMATION OF PLANTS AND PLANT CELLS**

Plants and plant cells may be transformed using any method known in the art. In an embodiment of the present invention, *Agrobacterium* is employed to introduce the gene construct into plants. Such transformation preferably uses binary *Agrobacterium* T-DNA vectors (Bevan, 1984, Nuc. Acid Res. 12:8711-8721), and the co-cultivation procedure (Horsch *et al.*, 1985, Science 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan *et al.*, 1982, Ann. Rev. Genet 16:357-384; Rogers *et al.*, 1986, Methods Enzymol. 118:627-641). The *Agrobacterium* transformation system may also be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells. (see Hernalsteen *et al.*, 1984, EMBO J 3:3039-3041 ; Hooykass-Van Slogteren *et al.*, 1984, Nature 311:763-764; Grimsley *et al.*, 1987, Nature 325:1677-179; Boulton *et al.*, 1989, Plant Mol. Biol. 12:31-40.; and Gould *et al.*, 1991, Plant Physiol. 95:426-434).

In other embodiments, various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells may also be utilized. These other methods are particularly useful where the target is a monocotyledonous plant or plant cell. Alternative gene transfer and transformation methods include, but are not limited to, particle gun bombardment (biolistics), protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski *et al.*, 1984, EMBO J 3:2717-2722, Potrykus *et al.* 1985, Molec. Gen. Genet. 199:169-177; Fromm *et al.*, 1985, Proc. Nat. Acad. Sci. USA 82:5824-5828; and Shimamoto, 1989, Nature 338:274-276) and electroporation of plant tissues (D'Halluin *et al.*, 1992, Plant Cell 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaeppeler *et al.*, 1990, Plant Cell Reporter 9:415-418), and microprojectile bombardment (see Klein *et al.*, 1988, Proc. Nat. Acad. Sci. USA 85:4305-4309; and Gordon-Kamm *et al.*, 1990, Plant Cell 2:603-618). In any methods, selectable markers may be used,

at least initially, in order to determine whether transformation has actually occurred. Useful selectable markers include enzymes which confer resistance to an antibiotic, such as gentamycin, hygromycin, kanamycin and the like. Alternatively, markers which provide a compound identifiable by a color change, such as GUS, or luminescence, such as luciferase, may be used.

The chimeric gene may also comprise a gene switch mechanism which determines under what conditions or when the coding sequence is to be expressed. The gene switch may be a chemically induced promoter or a temperature controlled promoter, for example.

According to the present invention, a wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the present invention and the various transformation methods mentioned above. In preferred embodiments, target plants and plant cells for engineering include, but are not limited to, those monocotyledonous and dicotyledonous plants, such as crops including grain crops (*e.g.*, wheat, maize, rice, millet, barley), fruit crops (*e.g.*, tomato, apple, pear, strawberry, orange), forage crops (*e.g.*, alfalfa), root vegetable crops (*e.g.*, carrot, potato, sugar beets, yam), leafy vegetable crops (*e.g.*, lettuce, spinach); flowering plants (*e.g.*, petunia, rose, chrysanthamum), conifers and pine trees (*e.g.*, pine fir, spruce); plants used in phytoremediation (*e.g.*, heavy metal accumulating plants); oil crops (*e.g.*, sunflower, rape seed) and plants used for experimental purposes (*e.g.*, *Arabidopsis*).

## **5.6 SCREENING OF TRANSFORMED PLANTS AND PLANT CELLS**

According to the present invention, desired plants may be obtained by engineering one or more of the gene constructs expressing a chitinase and  $\beta$ -1,3-glucanase as described herein into a variety of plant cell types, including but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollens, embryos, as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (those that have incorporated or integrated the introduced gene construct(s)) following the

approaches and methods described below. An isolated transformant may then be regenerated into a plant. Alternatively, the engineered plant material may be regenerated into a plant before subjecting the derived plant to selection or screening for the marker gene traits. Procedures for regenerating plants from  
5 plant cells, tissues or organs, either before or after selecting or screening for marker gene(s), are well known to those skilled in the art.

A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be  
10 performed by growing the engineered plant material on media containing inhibitory amount of the antibiotic or herbicide to which the transforming gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (*e.g.*, the  $\beta$ -glucuronidase, luciferase, B or C1 genes) that may be present on the  
15 recombinant nucleic acid constructs of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

Physical and biochemical methods may also be used to identify plant or plant cell transformants containing the gene constructs of the present invention. These methods include but are not limited to: 1) Southern analysis or PCR  
20 amplification for detecting and determining the structure of the recombinant DNA insert; 2) Northern blot, S1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme activity, where such gene products are encoded by the gene construct; 4) protein gel  
25 electrophoresis (PAGE), Western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for  
30 doing all these assays are well known to those skilled in the art.



## 5.7 TRANSGENIC PLANTS EXPRESSING AN ENGINEERED CHITINASE AND $\beta$ -1,3-GLUCANASE POLYNUCLEOTIDES

Transgenic plants are generated that express an engineered chitinase and  $\beta$ -1,3-glucanase gene. A transgenic plant expressing a chitinase and  $\beta$ -1,3-glucanase  
5 is less susceptible to the pathogenic effects of the pathogen of interest.

Transgenic plants may be made using any of the techniques known in the art as described for plant chitinase and  $\beta$ -1,3-glucanase expressing transgenic plants,  
*supra*.

Transgenic plants expressing one or more chitinase and  $\beta$ -1,3-glucanase gene  
10 polynucleotides capable of rendering said plants more resistant to a pathogen of interest may be from any plant species, plant genus, plant family, plant order, plant class, plant division of the kingdom of plants. *See, e.g.*, U.S. Patent Nos. 5,889,189; 5,869,720; 5,850,015; 5,824,842; PP10,742; PP10,704; PP10,682, which recite plant species, genres, families, orders, classes and divisions in  
15 which the chitinase and  $\beta$ -1,3-glucanase genes may be used.

Examples of plants are monocots, dicots, crop plants (*i.e.*, any plant species grown for purposes of agriculture, food production for animals including humans, plants that are typically grown in groups of more than about 10 plants in order to harvest for any reason the entire plant or a part of the plant, *e.g.*, a fruit, a flower  
20 or a crop, *e.g.*, grain, that the plants bear, etc.), trees (*i.e.*, fruit trees, trees grown for wood production, trees grown for decoration, etc.), flowers of any kind (*i.e.*, plants grown for purposes of decoration, for example, following their harvest), cactuses, etc.

Further examples of plants in which the chitinase and  $\beta$ -1,3-glucanase genes may  
25 be expressed include Viridiplantae, Streptophyta, Embryophyta, Tracheophyta, Euphyllophytes, Spermatophyta, Magnoliophyta, Liliopsida, Commelinidae, Poales, Poaceae, Oryza, Oryza sativa, Zea, Zea mays, Hordeum, Hordeum vulgare, Triticum, Triticum aestivum, Eudicotyledons, Core eudicots, Asteridae, Euasterids, Rosidae, Eurosids II, Brassicales, Brassicaceae, Arabidopsis,  
30 Magnoliopsida, Solananae, Solanales, Solanaceae, Solanum, Nicotiana.

- Also included are, for example, crops of particular interest including Solanaceae, including processing and fresh market tomatoes, pepper and eggplant; leafy plants, including lettuce and spinach; *Brassicas*, including broccoli, brussels sprouts, calabrese, kale, cauliflower, red cabbage and white cabbage; cucurbits, including cucumber, melon, watermelon, zucchini and squash; large seeded plants, including peas, beans and sweetcorn; rooted plants, including carrots and onions; vegetatively propagated plants, including berries, grapes, banana, pineapple and rosaceous fruit and nut crops; and tropical crops, including mango and papaya.
- Thus, the invention has use over a broad range of plants including, but not limited to, species from the genera *Anacardium*, *Arachis*, *Asparagus*, *Atropa*, *Avena*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Carthamus*, *Cocos*, *Coffea*, *Cucumis*, *Cucurbita*, *Daucus*, *Elaeis*, *Fragaria*, *Glycine*, *Gossypium*, *Helianthus*, *Heterocallis*, *Hordeum*, *Hyoscyamus*, *Lactuca*, *Linum*, *Lolium*, *Lupinus*, *Lycopersicon*, *Malus*, *Manihot*, *Majorana*, *Medicago*, *Nicotiana*, *Olea*, *Oryza*, *Panicum*, *Pennisetum*, *Persea*, *Phaseolus*, *Pistachia*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Ricinus*, *Secale*, *Senecio*, *Sinapis*, *Solanum*, *Sorghum*, *Theobromus*, *Trigonella*, *Triticum*, *Vicia*, *Vitis*, *Vigna*, and *Zea*.

## 5.8 POLYNUCLEOTIDE CONSTRUCTS FOR EXPRESSION OF ENGINEERED GENE IN TRANSGENIC PLANTS

- A polynucleotide construct capable of directing the expression of an engineered chitinase and  $\beta$ -1,3-glucanase gene product in a transgenic plant of interest is constructed using general recombinant DNA and cloning techniques known in the art of biotechnology, *see, e.g.,* Sambrook *et al., supra*; Ausubel *et al., supra*.
- Such a polynucleotide construct typically comprises a polynucleotide sequence that encodes an engineered chitinase and  $\beta$ -1,3-glucanase gene product and one or more regulatory polynucleotide sequence. Regulatory sequences useful for the polynucleotide construct of the invention include, but are not limited to, a promoter, an enhancer, an intron, a splice donor, a splice acceptor, a polyadenylation sequence, a RNA stability regulating sequence, or an element of

any one of the above (*e.g.*, promoter elements including, but not limited to, a TATA box).

The polynucleotide construct comprises one or more regulatory elements capable of directing the expression of the engineered chitinase and  $\beta$ -1,3-glucanase gene product of the invention. In a preferred aspect, the regulatory elements are capable of directing expression in a plant species in which expression of the engineered chitinase and  $\beta$ -1,3-glucanase gene product is desired. In another preferred aspect, the regulatory elements are capable of directing expression in a cell type in which expression of the engineered chitinase and  $\beta$ -1,3-glucanase gene product is desired in the plant species of interest.

Regulatory elements useful for the polynucleotide construct of the present invention are known to those of skill in the art, for example, promoter and enhancer elements of genes known to be expressed in the cell type and plant species of interest. A promoter useful for expression of the engineered chitinase and  $\beta$ -1,3-glucanase gene product in a cell type of a plant species of interest may also be isolated using routine experimentation, for example, by isolating a promoter region of a gene known to be expressed in the desired fashion. For example, one may screen a genomic library with a cDNA probe specific for the 5' end of a messenger RNA known to be expressed in the cell type of interest of the plant species of interest. Such a 5' end cDNA probe should preferably be only about 100 base pairs to about 300 base pairs so that the clones identified in the genomic library are likely to include the 5' end of the gene possibly including the promoter region of the gene for which the probe is specific. The promoter region typically includes about 1,000 to about 2,000 base pairs upstream of the transcription initiation site. Thus, a promoter useful for the expression of the engineered chitinase and  $\beta$ -1,3-glucanase genes of the present invention is a polynucleotide from about 2,000 base pairs upstream to about 50 base pairs downstream of the transcription initiation site of a gene known to be expressed in the cell type of interest in the plant species of interest, or is a portion of the polynucleotide.

In order to facilitate the proper processing of the engineered chitinase and  $\beta$ -1,3-glucanase gene product, it may be necessary to include a nucleotide stretch that encodes a peptide sequence necessary for such processing. For example, a peptide sequence which is recognized by and functional in the transgenic host plant, for example, to facilitate the entry of the chitinase and  $\beta$ -1,3-glucanase gene product into the endoplasmic reticulum may be necessary, *i.e.*, signal sequence.

### 5.9 ESSAYS FOR TESTING AN ENGINEERED RESISTANT PLANT LINE

Plant lines generated using methods of the present invention that express an engineered chitinase and  $\beta$ -1,3-glucanase gene product are more resistant to the pathogenic effects of a pathogen of interest when compared to a plant line of the same species that does not express the engineered chitinase and  $\beta$ -1,3-glucanase gene product (*i.e.*, a wild-type plant). The increased resistance of a plant line generated using methods of the invention may be assayed for by any technique known to the skilled artisan. For example, one may infect a plant of the generated plant line and a plant of a wild-type plant line with a pathogen of interest. After such infection, the plant of the generated plant line will have at least an approximately 20% higher probability of surviving infection than the wild-type plant, more preferably at least about 40%, more preferably at least about 60% and most preferably at least about 80%.

Another way of testing a transgenic plant made using the methods of the invention is by testing for necrosis inducing activity, for example, as described in Mahe *et al.*, 1998, J. Peptide Res. 52:482-494. Thus, one can express an engineered chitinase and  $\beta$ -1,3-glucanase gene in a transgenic plant and infect the transgenic plant with the pathogen of interest. For example, when applying a pathogen to the transgenic plant expressing the engineered chitinase and  $\beta$ -1,3-glucanase gene, one would observe clear necrosis or severe spreading necrosis in the wild-type plant but not in a transgenic plant of the plant line from which the transgenic plant was derived.

Necrotic cell death can also be observed using histochemical staining reactions in addition to visual inspection.

The following examples are provided to further illustrate the current invention but are not provided to in any way limit the scope of the current invention.

## 5 6. EXAMPLES

### 6.1 MATERIALS AND METHODS

#### *Plant material*

*Brassia juncea* plants were grown in a growth chamber at 22-24 °C under a 12 h light/12 h dark cycle.

#### 10 *Screening a B. juncea cDNA library for chitinase clones*

A pair of oligonucleotide primers, 5'GGTGGATGGGCTACAGCACCAGAC3' and 5'GCCACGTCCACACTCCAA3', were synthesized for PCR amplification of a 414 bp fragment (nucleotides 1625-2038) of the *Arabidopsis* chitinase gene (Samac *et al.*, 1990, Plant Physiol 93:907-914). The nucleotide sequence of  
15 this fragment which corresponds to a conserved chitinase domain was confirmed before use in screening a *B.juncea* cDNA library (Pua *et al.*, 1992, Plant Mol Biol 19:541-544) by *in situ* plaque hybridization at 42 °C in a solution containing 30% deionized formamide (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor,  
20 N.Y.). Blots were washed in 0.1 x SSC, 0.1% SDS at room temperature.

#### *DNA sequence analysis*

DNA fragments containing the sequences of interest in M13mpl8 (Yanisch-Perron *et al.*, 1985, Gene 33:103-119) were analyzed using the DNA Sequencing Kit with Sequenase Version 2.0 (UBS, Amersham Life Science) and the GCG  
25 sequence analysis program (Genetics Computer Group).

#### *Genomic DNA isolation and Southern blot analysis*

For Southern blot analysis, 20 µg DNA isolated (Dellaporta *et al.*, 1983, Plant Mol Biol Reprtr 1:19-21) from *B. juncea* was digested with various restriction endonucleases, separated by electrophoresis in 0.8% agarose gel and blotted onto Hybond-N (Amersham) (Sambrook *et al.*, 1989). The blot was hybridized to the <sup>32</sup>P-labelled *BjCH11* cDNA probe in a solution containing 30% deionized formamide, 6x SSC, 5x Denhardt's, 1% SDS and 500 µg/ml denatured, sonicated salmon sperm DNA at 42 °C overnight. The blot was washed in 0.1 x SSC, 0.1% SDS at room temperature and 65 °C.

#### *Northern blot analysis*

- 20µg of RNA extracted from leaves of seedlings (Nagy *et al.*, 1988, Plant Molecular Biology Manual p. B4:1-29. Kluwer Academic Publisher, Dordrecht) was denatured at 50 °C in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gel and blotted onto Hybond-N (Amersham) membranes. Blots were incubated with <sup>32</sup>P-labelled *BjCH11* cDNA in 50% formamide, 1 x Denhardt's, 6x SSPE, 0.1% SDS, 100 µg/ml denatured, sonicated salmon sperm DNA and 10% dextran sulfate at 42 °C overnight. The blots were washed in 0.1 x SSC, 0.1% SDS at 65 °C.

#### *Polymerase chain reactions*

- PCR with primers P1 and P2 or P2 and P3 was carried out with the GeneAmp PCR Reagent Kit (Perkin Elmer). Figure 1A shows the location of the primers: P1 (5'CCTCCGGTGAGCAATGCG3') corresponding to nucleotides 56 to 73, P2 (5'TTAGCGGCGGTGATGAAGG3') complementary to nucleotides 554 to 536 and P3 (5'TCCTCCAACCCCGCAGTGT3') corresponding to nucleotides 207 to 225. *B. juncea* DNA, denatured at 94 °C for 5 min, was subjected to PCR with primers for 35 cycles of 94 °C for 1 min, 68 °C for 1 min and 72 °C for 3 min. The final extension was carried out at 72 °C for 7 min. The PCR products electrophoresed in 2% agarose gel were blotted onto Hybond-N (Amersham) membrane for hybridization with the <sup>32</sup>P labelled *BjCH11* cDNA.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the protocol of Lasserre *et al.*, 1996, Mol Gen Genet 251:81-90, using total RNA extracted from leaves of seedlings using guanidine thiocyanate (Nagy *et al.*, 1988). Primer P4, 5'CCACTCGAGGTTGTTGC3'

- 5 (complementary to nucleotides 750 to 734; Figure 1A), was annealed to 50 µg total RNA. First-strand cDNA was used as template in PCR with primers P2 and P3 (Figure 1A). All RT-PCR reactions were done in triplicate.

#### *Preparation of polyclonal antibodies against HbGLU and BjCHI1*

- A synthetic peptide (SDLQSLTNPSNAKS) corresponding to amino acids 94-  
10 107 of HbGLU (SEQ ID NO:4) (Chye and Cheung, 1995, Plant Mol. Biol. 29:397-402) was purchased from Chiron Technologies (Australia) and was used to raise polyclonal antibodies in rabbit following Sambrook *et al.*, 1989. A synthetic peptide (YKEEIDKSDPHC) corresponding to amino acids 231-242 of BjCHI1 (SEQ ID NO:1) (Zhao and Chye, 1999, *supra*) was used for  
15 immunization of rabbit to raise polyclonal antibodies and these anti-BjCHI1 antibodies were purchased from Chiron Technologies (Australia). Each peptide was coupled to Keyhole Limpet Hemocyanin (KLH) and mixed with Freund's complete adjuvant for raising antibodies. Blood was collected and antibodies against HbGLU were purified using Protein A Sepharose CL-4B (Pharmacia)  
20 and CNBr-activated Sepharose 4B (Pharmacia) columns while antibodies against BjCHI1 were purified using a Thiopropyl-Sepharose 6B (Pharmacia) column.

#### *Infection of B. juncea with R. solani*

- B. juncea* seeds were sowed on autoclaved soil and were incubated in a growth chamber at 24°C with a day-night regime of 12 h light (08:00-20:00) and 12 h  
25 dark (20:00-08:00). Two week-old seedlings were carefully transferred to soil inoculated with a week-old culture of *R. solani*. The fungus was grown at room temperature for one week in potato dextrose broth and was added to the soil 3 days before the seedlings were planted. Leaves were harvested daily from

seedlings from 0 to 9 days after transfer to infected soil. Total RNA and total protein were extracted from these leaves for northern blot analysis and western blot analysis, respectively.

*Plasmid constructs that carries both the Hevea  $\beta$ -1,3-glucanase and Brassica chitinase BjCHI1*

A 1.2 kb *Sma*I-*Hind*II fragment of full-length *H. brasiliensis*  $\beta$ -1,3-glucanase cDNA, designated *HbGLU* (Chye and Cheung, 1995, Plant Mol. Biol. 29: 397-402) was cloned downstream from the CaMV 35S promoter in the *Sma*I site of binary plasmid vector pBI121 (Clontech) to generate plasmid pHEV43 (Figure 7) while a 1.3 kb *Sma*I fragment of full-length cDNA encoding *B. juncea* chitinase, BjCHI1 (Zhao and Chye, *supra*) was cloned downstream from the CaMV 35S promoter in the *Sma*I site of pBI121 (Clontech) to generate plasmid pBj17.

Subsequently from pHEV43, a 2.04 kb *Hind*III fragment containing the CaMV 35S promoter and the *HbGLU* cDNA was blunt-ended with Klenow and ligated to the *Sna*BI site within the GUS gene of pBj17, generating two plasmids pBj47 and pBj48 (Figure 7). The CaMV 35S promoters in pBj47 are inverted while those in pBj48 are in tandem and each of plasmids pBj47 and pBj48 carries both the *HbGLU* cDNA and the *BjCHI1* cDNA on a single plasmid (Figure 7).

Plasmids pBj17, pBj47 and pHEV43 were used in potato transformation.

## **6.2 GENERATION OF TRANSGENIC POTATO PLANTS CARRYING THE *HEVEA* $\beta$ -1,3-GLUCANASE AND *BRASSICA* CHITINASE BjCHI1 CONSTRUCTS**

### **Plant Transformation**

Each of plasmids pHEV43, pBj17, pBj47 and pBI121 was mobilized from *E. coli* strain DH5 $\alpha$  into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating using helper strain HB101 (pRK2013). Transformation of potato plant (*Solanum tuberosum* L.) variety Desiree by *A. tumefaciens* derivatives harboring one of these plasmids was carried out following the protocol of Dietze *et al*,



1995, In Potrykus I, Sprangenberg G (eds), Gene Transfer to Plants, New York, Cold Spring Harbor Laboratory, p. 24-29. Transformants were selected on Murashige and Skoog media supplemented with kanamycin (100 µg/ml).

Potato tissue cultures were maintained in a growth incubator under a 16 h light / 8 h dark regime at 20-25 °C. Transgenic R<sub>0</sub> potato plants were grown in soil in a growth chamber at 24 °C with a day/night regime of 12 h light /12 h dark.

### 6.3 TESTING THE FUNCTION OF *HEVEA* β-1,3-GLUCANASE AND *BRASSICA* CHITINASE BjCHI1 PRODUCTS IN STABLY TRANSFORMED POTATO PLANTS

#### 10 *Northern blot analysis*

Total RNA was extracted from whole plants following the method described by Nagy *et al.*, 1988, In: SV Gelvin, RA Schilperoort, (eds), Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, p.B4:1-29). Twenty (20) µg of RNA was denatured at 50°C for 30 min in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gel and transferred to Hybond-N membrane (Amersham). Blots were prehybridized for 4-6 h and then hybridized with <sup>32</sup>P-labeled BjCHI1 cDNA or HbGLU cDNA, prepared by random-primed labelling, in a solution containing 50% formamide, 1 x Denhardt's, 6 x SSPE, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA and 10% dextran sulphate at 42°C overnight. Blots were washed in 0.1 x SSC, 0.1% SDS at 65°C.

#### *Southern blot analysis*

For Southern blot analysis, 20 µg genomic DNA isolated according to Dellaporta *et al.*, 1983, Plant Mol Biol Rep. 1: 19-21, was digested with restriction endonucleases, separated by electrophoresis in 0.7% agarose gel and blotted onto Hybond-N (Amersham) membranes (Sambrook *et al.*, 1989). The membranes were prehybridized in a solution containing 50% deionized formamide, 6 x SSC, 5 x Denhardt's, 1% SDS, 100 µg/ml denatured salmon sperm DNA at 42°C for

4-6 h. The  $^{32}\text{P}$ -labeled *BjCHII* cDNA probe or  $^{32}\text{P}$ -labeled *HbGLU* cDNA probe was added and hybridized overnight. Membranes were washed in 0.1 x SSC, 0.1% SDS at 65 °C.

#### *Western blot analysis*

- 5 Total plant protein was prepared following the method described by Kush *et al.*, 1990, Proc Natl Acad Sci USA 87: 1787-1790. 20 µg of total protein was separated by SDS-PAGE and transferred onto Hybond-C (Amersham) membrane as described by Sambrook *et al.*, 1989. In western blot analysis, cross-reacting bands were detected using polyclonal antibodies against BjCHII  
10 or polyclonal antibodies against HbGLU following the procedures described in the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immunoblot Assay Kit (BioRad)

#### *Preparation of plant protein extracts for enzyme assays*

- Plant protein extracts were prepared according to Boller *et al.*, 1983, Planta  
15 157:22-31. Plants were ground to fine powder in liquid nitrogen, transferred to 0.1M Na-citrate (pH 5.0) buffer containing 1% (v/v) β-mercaptoethanol and vortexed. Following centrifugation (14,000 rpm for 5 min at 4°C), the supernatant was removed to another tube, incubated at 50°C for 10 min and cooled on ice for 10 min. The sample was then centrifuged (14,000 rpm for 5  
20 min at 4°C), and the supernatant was used as the crude protein in assays for chitinase and β-1,3-glucanase activities. Protein concentration was determined according to Bradford (1976, Anal. Biochem, 72:248-254).

#### *β-1,3-glucanase assays*

- Colorimetric assay for β-1,3-glucanase was carried out according to Abeles and  
25 Forrence, 1970, Plant Physiol 45:395-400. The reaction mixture of 100 µl (25 µg) plant protein extract and 100 µl of 2% (w/v) laminarin (Sigma) was incubated at 50°C for 2 h. The reaction was terminated by addition of 600 µl of dinitrosalicyclic reagent and heating for 5 min at 100°C. Following cooling to

room temperature, the contents were diluted 1:20 in water and the absorbance was measured at 500 nm.

### *Chitinase assays*

Colorimetric chitinase assays were carried out according to Wirth and Wolf, 1990, J. Microbiol Meth 12: 197-205. The reaction mixture of 150 µl of substrate carboxymethyl/chitin/Remazol Brilliant Violet (Loewe Biochemica; 2 mg/ml stock solution), 150 µl of 0.2 M Na-Acetate (pH 5.0) and 300 µl (15 µg) plant protein extract was incubated at 37°C for 0.5h. The reaction was terminated by addition of 150 µl of 1N HCl, followed by incubation on ice for 5-10 min, before centrifugation (14,000 rpm for 3 min at 4°C). The supernatant was used to read absorbance at 550 nm. For the blank, 300 µl of 0.1 M Na-citrate, pH 5.0 buffer was used instead of protein sample.

### *In vitro fungal bioassays using Tricoderma viride*

*T. viride* Persoon (ATCC 12582) was cultured on a plate of potato dextrose (Difco) agar (PDA). It was used for *in vitro* hyphal inhibition tests following Schlumbaum *et al.*, 1986, Nature 324:365-367. A plug of growing *T. viride* culture on PDA was transferred to the center of a fresh PDA plate. Following incubation at 25°C for 24 h during which the hyphae grew outwards from the center, wells were bored on the outer surface of the PDA, equidistant from the plug. Plant protein extract (50 µg) was added to each well and the plate was further incubated in the dark at 25°C after which growth inhibition of *T. viride* was observed. Photographs were taken at 16 h and 24 h.

### *In vivo bioassays using R. solani*

It was previously shown in northern blot analysis that *BjCH11* mRNA is induced by wounding or MeJA treatment (Zhao *et al.*, 1999, *supra*). Subsequently it was also shown that *BjCH11* is also induced by infection with *Aspergillus niger* infection and by caterpillar infestation (Fung *et al.*, 2002, *supra*). Here, *in vivo* bioassays were carried out on young potato plants according to Jach *et al.*, 1995,

Plant J. 8:97-109. *R. solani* incubated on solid PDA medium at 25°C for 5-6 days was inoculated into 100 ml of liquid potato dextrose broth (PDB) and incubated with shaking (100 rpm) at room temperature for 1-2 weeks.

Subsequently the culture was transferred to a conical flask containing 500 ml of fresh PDB and further incubated for 3-4 days. This culture was then thoroughly mixed with 6 L of sterilized soil (Bio-Mix Super, The Netherlands) in a plant growth tray containing sterilized 8 L sterilized soil. After 10 days the soil was uniformly mixed and potato plants, previously grown for 2 weeks in sterilized soil from tissue culture, were transplanted into the infected soil and further incubated in a growth chamber at 24°C under a 12 h light / 12 h dark regime. Photographs were taken of potato plants 2 weeks following transplantation to infected soil. Plants grown in sterilized soil without fungus was used as controls.

## 6.4 RESULTS

*BjCH11* expression is induced by *R. solani* infection

To investigate the effect of fungal induction on *BjCH11* expression, *B. juncea* seedlings were grown in soil preinoculated with the soil fungus, *R. solani*. Our results on Figure 8a demonstrate that expression of the 1.3 kb *BjCH11* mRNA increases a day following growth in infected soil. Figure 8b shows that a cross-reacting band with an apparent molecular mass of 37 kDa expected of the mature *BjCH11* protein accumulates from day 3 following growth in infected soil. The 42-kDa faint band above this band is likely a precursor protein (Figure 8b). The *BjCH11* precursor of calculated molecular mass 42,774 kDa, undergoes post-translational cleavage whereby an N-terminal signal peptide and a C-terminal vacuolar targeting peptide are removed (Zhao and Chye, *supra*). Our results suggest that *BjCH11* mRNA and its corresponding protein accumulate following *R. solani* infection, implicating its role in fungal defense.

*Expression of BjCH11 and HbGLU in transgenic potato plants*

The observation that *BjCHII* expression is induced by *R. solani* infection led us to investigate if its heterologous expression in transgenic potato plant could confer protection against *R. solani*. *BjCHII* cDNA-containing plasmids, pBj17 and pBj47, were each introduced into potato variety Desiree by *A. tumefaciens*-mediated transformation. Plasmid pBj47 also contains the *HbGLU* cDNA, facilitating us to investigate the effect, if any, of *HbGLU* in enhancing *BjCHII* action. Plasmid pHEV43 that contains the *HbGLU* cDNA alone and vector control, pBI121, were also used in potato transformation. About twenty independent kanamycin-resistant putative transgenic plants from each transformation were examined by northern, Southern and western blot analyses. The results of analyses of the representative lines from each transformation that were eventually chosen for experiments of *R. solani* infection, are shown in Figures 9, 10 and 11. Figure 9A shows the presence of a 1.2 kb hybridizing *HbGLU* mRNA detected using <sup>32</sup>P-labelled *HbGLU* cDNA in northern blot analysis of transgenic potato lines co-expressing *HbGLU* and *BjCHII*, pBj47-P<sub>10</sub> (lane 2), pBj47-P<sub>8</sub> (lane 3), pBj47-P<sub>7</sub> (lane 4) and transgenic potato line expressing *HbGLU* alone, pHEV-P<sub>14</sub> (lane 5). This band is absent in untransformed potato (Figure 9A, lane 1). Figure 9B shows the expression of the 1.3 kb *BjCHII* hybridizing mRNA as detected with a <sup>32</sup>P-labeled *BjCHII* cDNA probe in transgenic potato lines pBj47-P<sub>10</sub> (lane 2), pBj47-P<sub>8</sub> (lane 3), pBj47-P<sub>7</sub> (lane 4) and pBj17-P<sub>6</sub> (lane 6). This band is absent in untransformed potato (Figure 9B, lane 1) and in pBI121 transformed potato (Figure 9B, lane 5).

#### *Southern blot analysis on transgenic potato lines*

Subsequently, DNA from these transgenic potato lines was used in Southern blot analysis with <sup>32</sup>P-labeled probes prepared from *BjCHII* or *HbGLU* cDNA. Results in Figure 10A show that a 1.2 kb *HbGLU* *EcoRI*-hybridizing band is present in transgenic potato lines pHEV43-P<sub>14</sub> (lane 3), pBj47-P<sub>7</sub> (lane 4), pBj47-P<sub>8</sub> (lane 5) and pBj47-P<sub>10</sub> (lane 6) and is absent in both untransformed potato (lane 1) and in pBI121 transformed potato (lane 2). The 1.2 kb *EcoRI*-

hybridizing band corresponds to that shown in the map of pHEV43 (Figure 7) and contains the full-length *HbGLU* cDNA. Using a  $^{32}\text{P}$ -labeled *BjCH11* cDNA probe and *Hind*III-digested DNA in Southern blot analysis (Figure 10B), a 0.9 kb *Hind*III-hybridizing band was detected in transgenic potato lines pBj47-P<sub>7</sub> (lane 3), pBj47-P<sub>8</sub> (lane 4), pBj47-P<sub>10</sub> (lane 5) and pBj17-P<sub>6</sub> (lane 6). This 0.9 kb band corresponds to the fragment between the second and third internal *Hind*III sites within the *BjCH11* cDNA as shown in the map of pBj17 (Figure 7). This 0.9 kb hybridizing band was absent in untransformed potato (Figure 10B, lane 1) and in pBI121 transformed potato (lane 2). When *Eco*RI-digested DNA was probed with a  $^{32}\text{P}$ -labeled *BjCH11* cDNA probe in Southern blot analysis (Figure 10C), hybridizing bands of various sizes were seen with transgenic potato lines pBj47-P<sub>10</sub> (lane 3), pBj47-P<sub>8</sub> (lane 4) and pBj47-P<sub>7</sub> (lane 5) suggesting that they are independent transgenic lines. Such hybridizing bands were absent in untransformed potato (lane 1) and in pBI121 transformed potato (lane 2).

#### *Protein detection of BjCH11 and HbGLU in transgenic potato*

Transgenic plants were further analyzed by western blot analysis using the anti-HbGLU antibodies and anti-BjCH11 antibodies. Western blot analysis using anti-HbGLU antibodies (Figure 11A) on crude protein from transgenic potato lines pBj47-P<sub>7</sub> (lane 1), pBj47-P<sub>8</sub> (lane 2), pBj47-P<sub>10</sub> (lane 3) and pHEV43-P<sub>14</sub> (lane 4) show a cross-reacting band with an apparent molecular mass of 35 kDa corresponding to HbGLU. This band was absent in pBI121 transformed potato (Figure 11A, lane 5) and in untransformed potato (lane 6). Western blot analysis using anti-BjCH11 antibodies (Figure 11B) on crude protein from transgenic potato lines pBj47-P<sub>7</sub> (lane 1), pBj47-P<sub>8</sub> (lane 2), pBj47-P<sub>10</sub> (lane 3), pHEV43-P<sub>14</sub> (lane 4), pBI121 transformed potato (lane 5), untransformed potato (lane 6) and transgenic potato line pBj17-P<sub>6</sub> (lane 7) shows a cross-reacting BjCH11 band, with an apparent molecular mass of 52 kDa, in transgenic potato lines pBj47-P<sub>7</sub> (lane 1), pBj47-P<sub>8</sub> (lane 2), pBj47-P<sub>10</sub> (lane 3) and pBj17-P<sub>6</sub> (lane

7). This band was absent in the transgenic line expressing HbGLU alone, pHEV43-P<sub>14</sub> (Figure 11B, lane 4), in pBI121 transformed potato (Figure 11B, lane 5) and in untransformed potato (Figure 11B, lane 6). The apparent molecular mass (52 kDa) of potato-expressed BjCHI1 resembles that observed for tobacco-expressed BjCHI1 (Fung *et al.*, 2002, *supra*), and is larger than that of native BjCHI1 (37 kDa), likely attributed to the inability of proper post-translational processing in heterologous hosts, tobacco and potato.

#### *Chitinase and $\beta$ -1,3-glucanase activity assay*

Transgenic potato lines were tested for chitinase and  $\beta$ -1,3-glucanase activity assays and were compared to pBI121 transformed potato (Figure 12). Glucanase assays (Figure 12A) using crude protein from pBI121-transformant and transgenic potato lines pBj47-P<sub>7</sub>, pBj47-P<sub>8</sub> and pBj47-P<sub>10</sub> show that the transgenic lines had higher levels of  $\beta$ -1,3-glucanase activity than that of the pBI121 transformant. Chitinase assays (Figure 12B) using crude protein show that transgenic potato lines pBj47-P<sub>7</sub>, pBj47-P<sub>8</sub> and pBj47-P<sub>10</sub> show higher levels of chitinase activities than of the pBI121 transformant. Activities detected in the pBI121 transformant are due to the presence of endogenous potato  $\beta$ -1,3-glucanases and chitinases.

#### *In vitro bioassay using *T. viride**

Subsequently crude extracts from transgenic potato lines were tested for *in vitro* inhibition of *T. viride* growth. Extracts from untransformed potato and a transgenic line transformed with pBI121 were used as controls in these bioassays with buffer as a blank. Photographs taken 16 h (Figure 13A) and 24 h (Figure 13B) after addition of protein extracts to wells show that the growth of *T. viride* was inhibited by extract from transgenic potato line pBj47-P<sub>10</sub> that co-expresses BjCHI1 and HbGLU while inhibition was absent in the buffer-only control (well 2), in extracts from wild-type potato (well 3) and pBI121 transformed potato (well 4). Transgenic potato lines pBj17-P<sub>6</sub> expressing BjCHI1 only (well 5)

and pHEV43-P<sub>14</sub> expressing  $\beta$ -1, 3-glucanase only (well 6) show weaker inhibition than that seen with pBj47-P<sub>10</sub> (well 1).

*In vivo bioassay using R. solani*

*In vivo* bioassays were carried out using young plants grown on soil

- 5 preinoculated with *R. solani*. Results documented two weeks after transfer to infected soil show that the transgenic potato line pBj47-P<sub>10</sub> (Figure 14A) that co-expresses BjCHI1 and HbGLU had slightly better growth than transgenic potato line pBj17-P<sub>6</sub> (Figure 14B) expressing BjCHI1 only. Root development in transgenic potato lines pBj47-P<sub>10</sub> and pBj17-P<sub>6</sub> were much better than that of
- 10 transgenic potato line pHEV43-P<sub>14</sub> (Figure 14C) expressing HbGLU only. Nonetheless root development of transgenic line pHEV43-P<sub>14</sub> was better than that of untransformed potato (Figure 14D) suggesting that expression of HbGLU *per se* conferred some protection. The control plants grown in sterilized soil (not inoculated with *R. solani*) showed root development similar to the pBj47-P<sub>10</sub>
- 15 transgenic plants (data not shown). This *in vivo* bioassay experiment using *R. solani* was repeated with consistent results.

- The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the
- 20 scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. Various references are cited herein, the disclosure of which are incorporated by
- 25 reference in their entirety.



WHAT IS CLAIMED IS:

1. A recombinant vector comprising: (a) a nucleotide sequence of SEQ ID NO:1 or a nucleotide sequence that encodes the amino acid sequence of SEQ ID NO:2 ;  
5 and (b) a nucleotide sequence of SEQ ID NO:3 or a nucleotide sequence that encodes the amino acid sequence of SEQ ID NO:4.
2. A recombinant vector comprising: (a) a first nucleotide sequence that hybridizes under stringent conditions to the complement of the nucleotide sequence of SEQ ID NO:1; and (b) a second nucleotide sequence hybridizes  
10 under stringent conditions to the complement of the nucleotide sequence of SEQ ID NO:3; wherein the stringent conditions comprise hybridization in 50% deionized formamide, 6x SSC, 5x Denhardt's, 1% sodium dodecyl sulfate (SDS), 100 µg/ml denatured 50% deionized formamide, 6xSSC, 5x Denhardt's, 1% sodium dodecyl sulfate (SDS), 100 µg/ml denatured salmon sperm DNA at 42°C,  
15 and washing in 0.1xSSC/0.1% SDS at 65°C; and wherein the first nucleotide sequence encodes a functional chitinase and the second nucleotide sequence encodes a functional  $\beta$ -1,3-glucanase.
3. A recombinant vector comprising: (a) a first nucleotide sequence that encodes a chitinase that comprises more than one chitin-binding domain; and (b) a second  
20 nucleotide sequence that encodes a  $\beta$ -1,3-glucanase.
4. The recombinant vector of claim 1, 2, or 3 further comprising one or more regulatory nucleic acid operatively linked to the first nucleotide sequence and the second nucleotide sequence.
5. The recombinant vector of claim 4 wherein the regulatory nucleic acid is 35S  
25 promoter of cauliflower mosaic virus.
6. A recombinant cell comprising the vector of claim 1, 2, or 3.
7. A recombinant cell comprising the vector of claim 4.

8. The recombinant cell of claim 6 wherein the cell is a plant cell.
9. The recombinant cell of claim 7 wherein the cell is a plant cell.
10. The recombinant cell of claim 8 wherein the plant is selected from the group consisting of wheat, maize, rice, barley, tomato, apple, pear, strawberry, carrot,  
5 potato, sugar beets, yam, lettuce, and spinach.
11. The recombinant cell of claim 9 wherein the plant is selected from the group consisting of wheat, maize, rice, barley, tomato, apple, pear, strawberry, carrot, potato, sugar beets, yam, lettuce, and spinach.
12. The recombinant cell of claim 10 wherein the plant is *Solanum tuberosum*.
- 10 13. The recombinant cell of claim 11 wherein the plant is *Solanum tuberosum*.
14. A recombinant cell comprising: (a) a first recombinant vector that comprises a first nucleotide sequence that encodes a chitinase that comprises more than one chitin-binding domain; and (b) a second recombinant vector that comprises a second nucleotide sequence that encodes a  $\beta$ -1,3-glucanase.
- 15 15. A recombinant cell comprising: (a) a first recombinant vector that comprises a first nucleotide sequence that hybridizes under stringent conditions to the complement of the nucleotide sequence of SEQ ID NO:1; and (b) a second recombinant vector that comprises a second nucleotide sequence hybridizes under stringent conditions to the complement of the nucleotide sequence of SEQ  
20 ID NO:3; wherein the stringent conditions comprise hybridization in 50% deionized formamide, 6x SSC, 5x Denhardt's, 1% sodium dodecyl sulfate (SDS), 100  $\mu$ g/ml denatured salmon sperm DNA at 42°C, and washing in 0.1xSSC/0.1% SDS at 65°C; and wherein the first nucleotide sequence encodes a functional chitinase and the second nucleotide sequence encodes a functional  $\beta$ -1,3-  
25 glucanase.

16. The method for producing a transformed plant which is resistant to pathogens comprising: (a) transforming a plant with a recombinant vector comprising: (i) a first nucleotide sequence that hybridizes under stringent conditions to the complement of the nucleotide sequence of SEQ ID NO:1; and  
5 (ii) a second nucleotide sequence hybridizes under stringent conditions to the complement of the nucleotide sequence of SEQ ID NO:3; wherein the stringent conditions comprise hybridization in 50% deionized formamide, 6x SSC, 5x Denhardt's, 1% sodium dodecyl sulfate (SDS), 100 µg/ml denatured salmon sperm DNA at 42°C, and washing in 0.1xSSC/0.1% SDS at 65°C ; and wherein  
10 the first nucleotide sequence encodes a functional chitinase and the second nucleotide sequence encodes a functional  $\beta$ -1,3-glucanase; and (b) selecting a transformed plant wherein the first and second nucleotide sequence are expressed.

17. A method for producing a transformed plant which is resistant to pathogens  
15 comprising: (a) transforming a plant with a first recombinant vector comprising a first nucleotide sequence that hybridizes under stringent conditions to the complement of the nucleotide sequence of SEQ ID NO:1; (b) transforming a plant with a second recombinant vector comprising a second nucleotide sequence hybridizes under stringent conditions to the complement of the  
20 nucleotide sequence of SEQ ID NO:3; wherein the stringent conditions comprise hybridization in 50% deionized formamide, 6x SSC, 5x Denhardt's, 1% sodium dodecyl sulfate (SDS), 100 µg/ml denatured salmon sperm DNA at 42°C, and washing in 0.1xSSC/0.1% SDS at 65°C ; and wherein the first nucleotide sequence encodes a functional chitinase and the second nucleotide sequence  
25 encodes a functional  $\beta$ -1,3-glucanase; and (c) selecting a transformed plant wherein the first and second nucleotide sequence are expressed.

18. The method of claim 16 or 17 wherein the pathogen is fungus.

19. The method of claim 18 wherein the fungus is *R. solani*.

**P1→**

aacatgaagacttatctccttctccttctcatctctccattctcttcttatacttttctccgggtgagcaatcggttagtcaatccataaccggaggagcactctgcccaacgggtctatgc 120  
M K T Y L L L L L L S F S S G E A Q C G S Q S I P E G A L C P N G L C 39

**HindIII**

tgcagcagggttggtggtggcgccaccacgaaagtcttactgcgggcctggttgtcacaagccagtgcaatccgttcctctatcctctccctccaaccccagctgggtcgctcaatccata 240  
C S E A G W C G T T E A Y C G H G C Q S Q C N P G P Y P P P P T T P Q C G R Q S I 79

**P3→**

ccccggggagccctctgcccaacgggtctatgctgtagcaggctggtggtggcgccaccacgaaagtcttactgcgggcatggttgcccaagccagtgcaactcccactcccactcctct 360  
P A G A L C P N G L C C S E A G W C G T T E A Y C G H G C Q S Q C T P T P T P P 119

**HindIII**

gtcccaactcccactcctcctactcccactcctcctagctcctacctcctggtcccactcctcctggtcccaggggatcttctggcatacttcaagagatcagtttetatatgaatg 480  
A P T P T P P T P P T P P S P T P P P T P P G P S G D L S G I I S R D Q F Y K M 159

cctaagcacatgaacgacaatgattgtcagtctgtgggtttcttctcacttacgacgcctctdtaaccggcggtatgctttcccaagtttcgggaacaccggagaccttgcctatgaggaag 600  
L K H M N D N D C H A V G F F T Y D A F I T A A K S F P S F G N T G D L A H R K 199

**←P2**

aaggagatagcagccttcttcggccagacttcccacgaaacacccggtgggtgggtgcacccgactggagcaaatatcaggggctactgtttacagaagaaaattgacaaaaagcgtat 720  
K E I A A F F G Q T S H E T T G G W S G A P D G A N T W G Y C Y K E E I D K S D 239

cctccactgtgatgcaacaacccctagctgctgcgcaccaggcacaattttactacggacgggaaccaatgatgctgcttctggaaactataattacggaccggtgcgggagagacctagga 840  
P H C D S N N L E W P C A P G K F Y Y G R G P H M L S W N Y N Y G P C G R D L G 279

**←P4**

ctcgagtactcaagaaccagatgttgctgcgcacccagtgatagctttcaaacccgccatttggtttctggatgactcctcaagctcctaataacccctcgtgccacgacgtgatcacc 960  
L E L L K N P D V A S S D P V I A F K T A I W F W M T P Q A P K P S C H D V I T 319

gaccagtgggagccgtcggtgcgcacatttctgcgggaaggttaccagggttatggagtgattaccaatatcatcaacggtggattagagtggtgctggtgcgcagctgcgaaggtccaa 1080  
D Q W E P S A A D I S A G R L P G Y G V I T N I I N G G L E C A G R D V A K V Q 359

gacgggatatcgctttttatcaaggtactgtggcattgttggtgtgatcctggaaagtaatttgactgtgacaatcaaaggccgtttaatgaaggtagtaacgttttcttggatgctgca 1200  
D R I S F Y T R Y C G H F G V D P G S N I D C D N Q R P F N E G S N V F L D A A 399

**HindIII**

atttaataagctactgttaatgagcttttctgtatccaagcaataagagagatcaaatataaattaataataaacctcttttcttaagtaaaaaaaaa 1297  
I \* 400

FIG. 1A

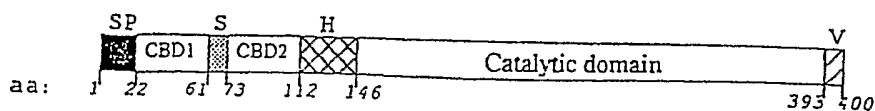


FIG. 1B



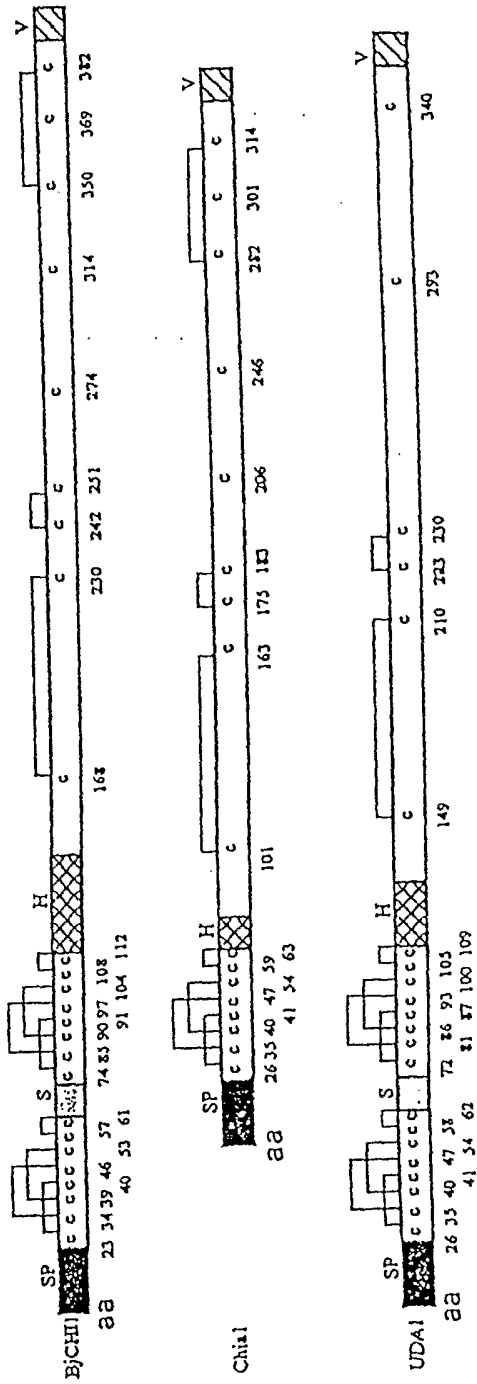


FIG. 2B

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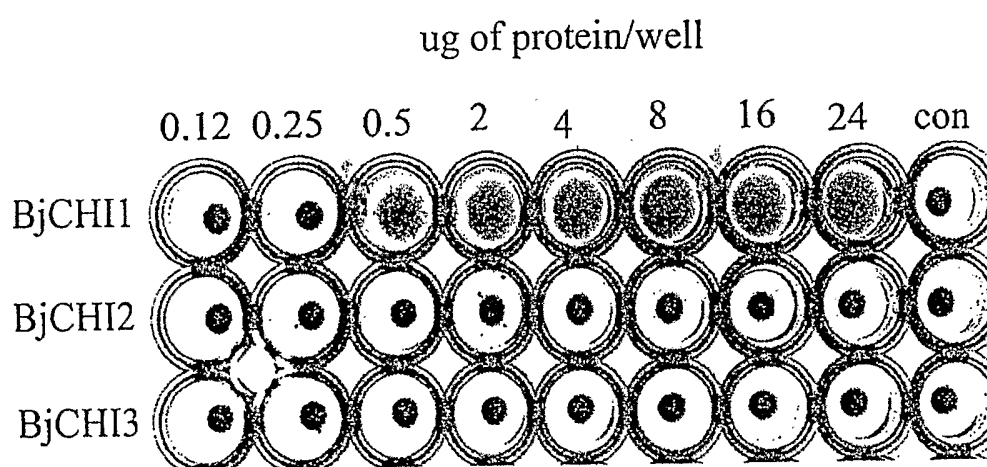


FIG. 3

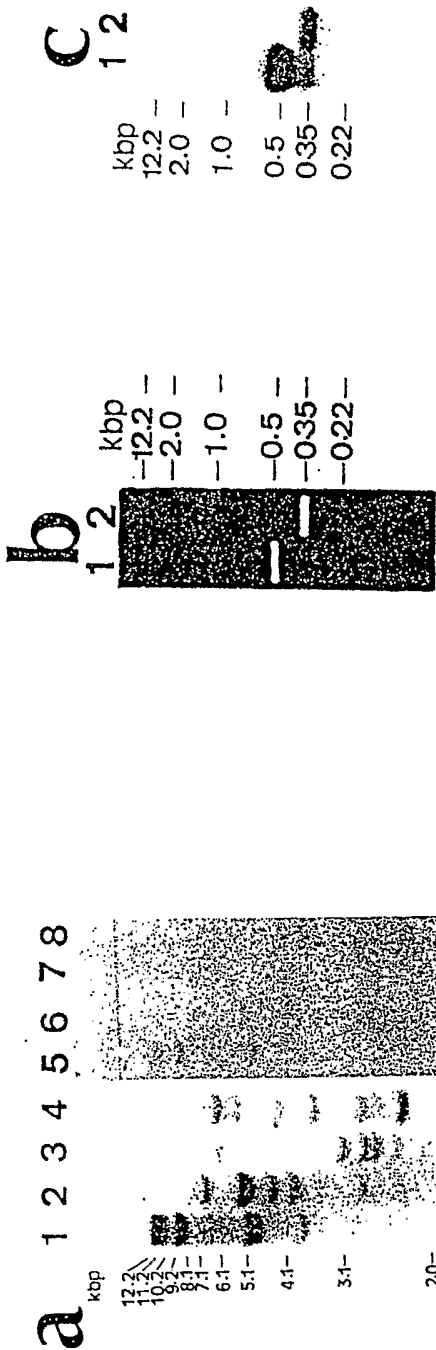


FIG. 4B

FIG. 4C



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AAATTATAAG	CAACTTTCTT	CTAATTTCCC	CCCTTCTTAA	TGGCTATCTC	CTCTTCAACT
TCAGGAACTA	GTAGTTCCTT	CCCCTCAAGA	ACTACTGTCA	TGCTTCTTCT	GTTTTTCTTT
GCAGCAAGCG	TTGGTATAAC	AGATGCCCAG	GTAGGTGTTT	GCTATGGAAT	GCAAGGCAAC
AACCTTCCAC	CTGTTTCAGA	GGTCATAGCT	CTCTATAAAA	AATCTAACAT	CACGAGAATG
AGAATTTATG	ATCCAAATCG	AGCAGTATTG	GAAGCCCTTA	GAGGCTCAAA	CATTGAACTC
ATACTAGGTG	TTCCAAACTC	AGATCTCCAA	AGCCTTACCA	ATCCTTCCAA	TGCAAAATCA
TGGGTACAAA	AAAATGTTCC	TGGCTTCTGG	TCAAGTGTCC	TGTTTCAGATA	TATAGCAGTT
GGCAACGAAA	TTAGTCCTGT	CAATAGAGGC	ACAGCTTGGT	TGGCTCAATT	TGTTTTGCCT
GCCATGAGAA	ATATACATGA	TGCTATAAGA	TCAGCTGGTC	TTCAAGATCA	AATCAAGGTC
TCCACTGCAA	TTGACTTGAC	CCTGGTAGGA	AATTCCTACC	CTCCTTCTGC	AGGTGCTTTC
AGGGATGATG	TTAGATCATA	CTTGGACCCA	ATTATTGGAT	TTCTATCCTC	TATCAGGTCA
CCTTTACTTG	CCAATATTTA	TCCTTACTTT	ACTTATGCTT	ATAATCCAAG	GGATATTTCC
CTTCCCTATG	CTTTGTTTAC	TTCACCATCA	GTTGTTGTGT	GGGATGGTCA	GCGAGGTTAT
AAGAACCTTT	TTGATGCAAC	GTTGGATGCA	TTGTACTCTG	CTCTTGAGAG	GGCTAGTGGT
GGTTCTCTGG	AGGTGGTTGT	TTCGGAAAGT	GGCTGGCCGT	CTGCCGGAGC	ATTTGCTGCC
ACATTTGACA	ATGGGCGTAC	TTATCTCTCA	AATTTGATCC	AACATGTTAA	AGGAGGTACT
CCTAAGAGGC	CTAACAGAGC	TATAGAGACT	TACTTATTTG	CCATGTTTGA	TGAAAATAAG
AAGCAACCAG	AGGTTGAGAA	ACACTTTGGA	CTTTTCTTTC	CTGATAAACG	GCCAAAATAT
AATCTCAATT	TTGGTGCAGA	AAAGAACTGG	GATATTTCTA	CTGAACACAA	TGCAACAATA
CTTTTCCTTA	AGAGTGATAT	GTGAGATTGT	GAGAATTTAA	GTACTATATA	TATTTCCAAT
GTATGCATGT	ATCCATGTAT	TAAATAAGAG	AACCTTTTCT	CA	

FIG. 5A

MAISSSTSGT	SSSFPSRTTV	MLLLFFFAAS	VGITDAQVGV	CYGMQGNLNP	PVSEVIALYK
KSNITRMRIY	DPNRAVLEAL	RGSNIELILG	VPNSDLQSLT	NPSNAKSWVQ	KNVRGFWSSV
LFRYIAVGNE	ISPVNRGTAW	LAQFVLPAMR	NIHDAIRSAG	LQDQIKVSTA	IDLTLVGNSY
PPSAGAFRDD	VRSYLDPIIG	FLSSIRSPLL	ANIYPYFTYA	YNPRDISLPY	ALFTSPSVVV
WDGQRGYKNL	FDATLDALYS	ALERASGGSL	EVVVSESGWP	SAGAFATFD	NGRTYLSNLI
QHVKGKTPKR	PNRAIETYLE	AMFDENKKQP	EVEKHFGGLFF	PDKRPKYNLN	FGEKNWDIS
TEHNATILFL	KSDM				

FIG. 5B

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Hb MAISSSTSGTS-SSFPSRTTVMLLFFFAASVGITDAQ--VGVCYGMQGN<sup>47</sup>  
 Np MD,.HKHIALQMAAII..GLLVS.TE.VG..S-.....L..  
 CII M.LCIK-NG.L--AAALV.VGLLIC.IQMIG..S-I.....KHA.  
 CIII MAHLIV.LL..SVLTL.TLDF.G.--A.....R...  
 CIV MALWYLFNKR.LGA--A.LI.VGLLMCNIQM.G..SNI.....KIA.  
 Hb NLPPVSEVIALYKKSNIITRMRIYDPNRAVLEALRGSNIELILGVPNSDIQ<sup>97</sup>  
 Np ....A.Q.VQ...SK..R...L....Q.A.Q.....VM.....  
 CII ...SDQD..N..NANG.RK....N.DTN.FN.....I..D..LQ...  
 CIII G..SPAD.VS.CNRN..R.....DQPT.....M.....P..E  
 CIV ...SEQD..N...ANG.RK....NSDTNIFKS.N.....I..D...Q..E  
 Hb SL-TNPSNAKSWVQKNVRGEWSSVLFRIYAVGNEISPVNRGTAWLAQFVL<sup>146</sup>  
 Np NIAA.....NN...R...N..PA.K.....V...T...SS.TRYL.  
 CII ...D..R.NG...D.IINHFPD.K.K.....V..G.N.Q--Y.P..A  
 CIII NVAASQA..DT...N...NY-GN.K.....V..L.ENSKYVP-VL.  
 CIV A.-A.S.I.NG...D.I.SHEPY.K.K..SI...V..S.N.Q--YS..L.  
 Hb PAMRNIHDAIRSAGLQDQIKVSTAILTLVGNSYPPSAGAFRDDVRSYLD<sup>196</sup>  
 Np .....RN..S.....NN....SSV.M..I...F...Q.S..N...FI.  
 CII ..Q.VYN.LAA.....TYSGILA.T...KDSI..GEFN.FIN  
 CIII N.....QT..SG...GN.....ETG.TTDS...N.R.K...QFIE  
 CIV H..E.VYN.LAA.....K...T..TYSG.LA.T...KDSI..EEFK.FIN<sup>246</sup>  
 Hb PIIGFLSSIRSPLANIYPYFTYAYNPRDISLPYALFTSPSVVWDGQRG<sup>246</sup>  
 Np .....VRR.N...V.....S..G.....A.N...Q'.SL.  
 CII ...Q..VQHNL.....V....GHIF.TA.VP.S....QQE---ANPA.  
 CIII ...N..VTN.A...V.L...AI.N.A-.K.E.....SE...N.NG..  
 CIV ...E..ARNNL.....GHI..TV.VP.S....NQQG---TNST.  
 Hb YKNLFDATLDALYSALERASGGSLVVVSESGWPSAGAFAT-FDNGRTY<sup>295</sup>  
 Np .R....MS..V.A..S..G...I.I.....-TN.AA..  
 CII .Q.....L..SM.F.V.K.G.QNV.II.....E.NS..T-IE.AQ..  
 CIII .R.....I...T....K...S...I.....GQL.SI..A..  
 CIV .Q.....L..SI.F.V.K.G.PNV.II.....E.NS...-IE.AQ..  
 Hb LSNLIQHVK--GGTPKRPNRAIETYLAFMFENKKQPEV-EKHFGLEFFPD<sup>342</sup>  
 Np YK.....--R.S.R...KV.....N.N..L.....S.N  
 CII YE...N...SGA....K.GK.....N.EGDIT.....S..  
 CIII NN...S...--S...SGP....V..L...DQ.D..I-.....SAN  
 CIV YR..VN...GGA....K.G.IV.....E.NG..T.....Y.N  
 Hb KRPKYNLNFG-AEKNWDISTERNATILFLKSDM<sup>374</sup>  
 Np .Q...P.S..FSDRY....A.N...AAS.I.E..  
 CII Q.A..Q...N  
 CIII MQ...QIS.N  
 CIV RTA..Q...MYS

FIG. 5C

8/16

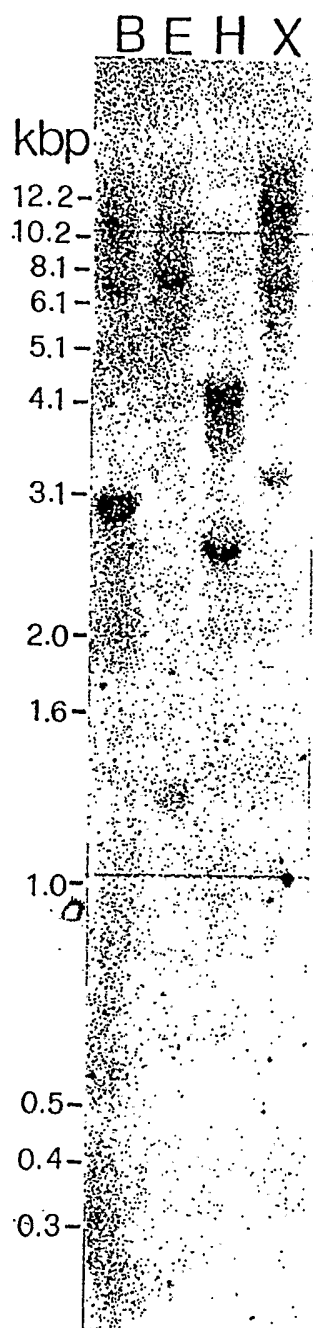


FIG. 6

9/16

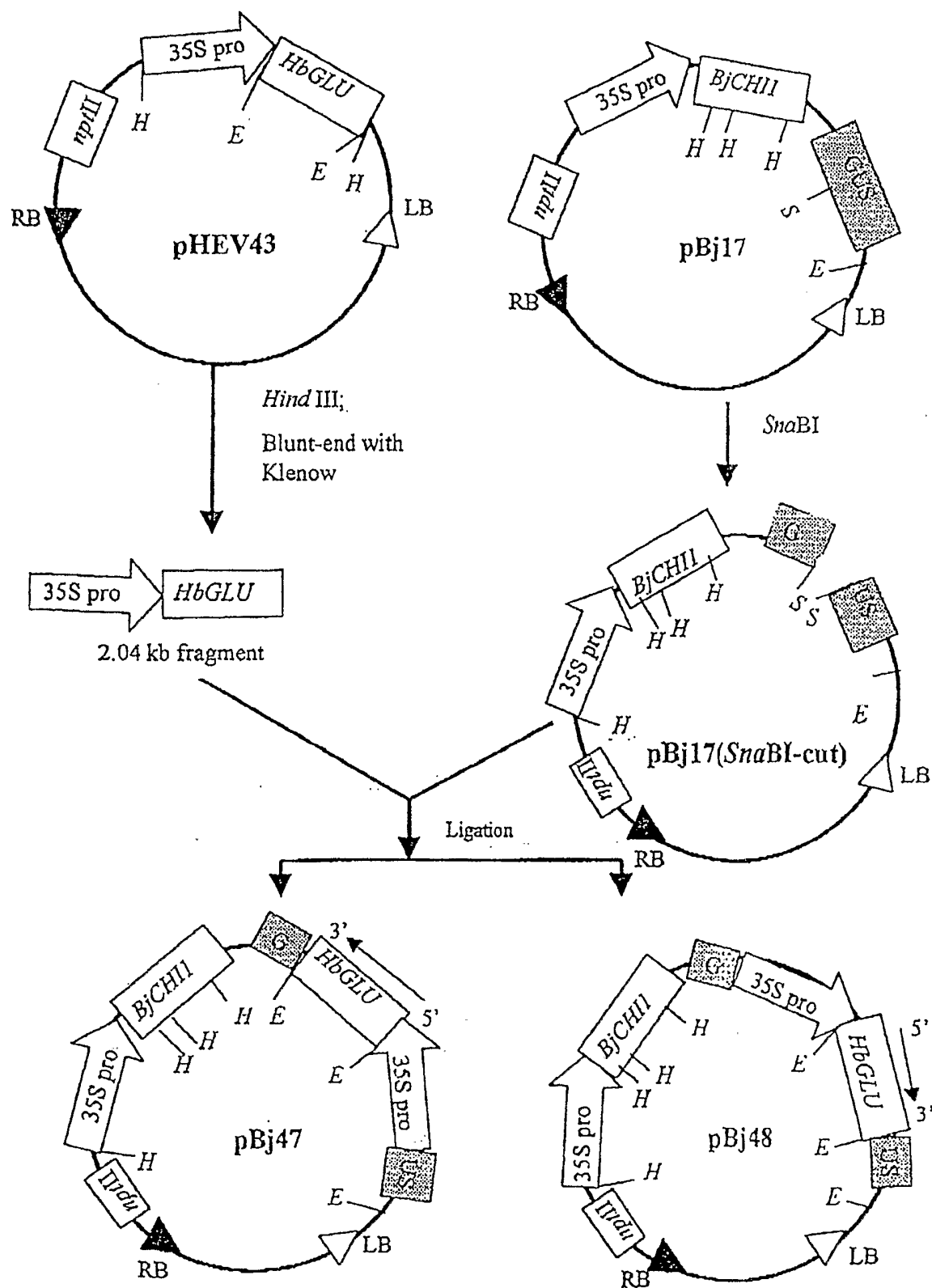


FIG. 7

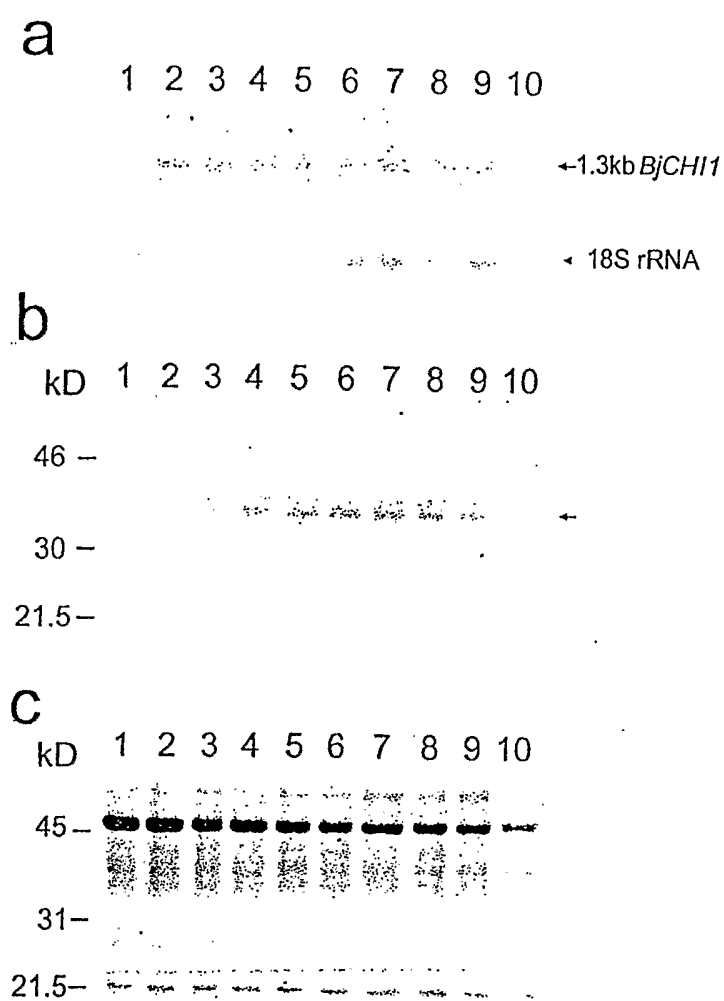


FIG. 8

11/16

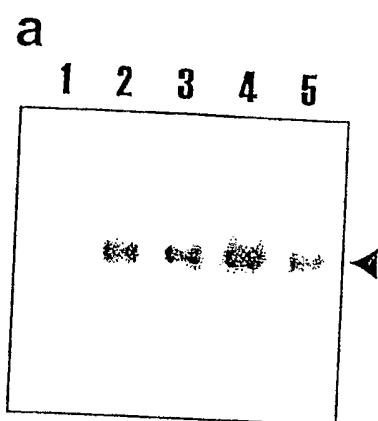


FIG. 9A

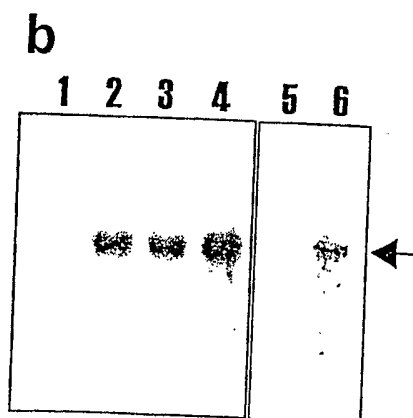


FIG. 9B

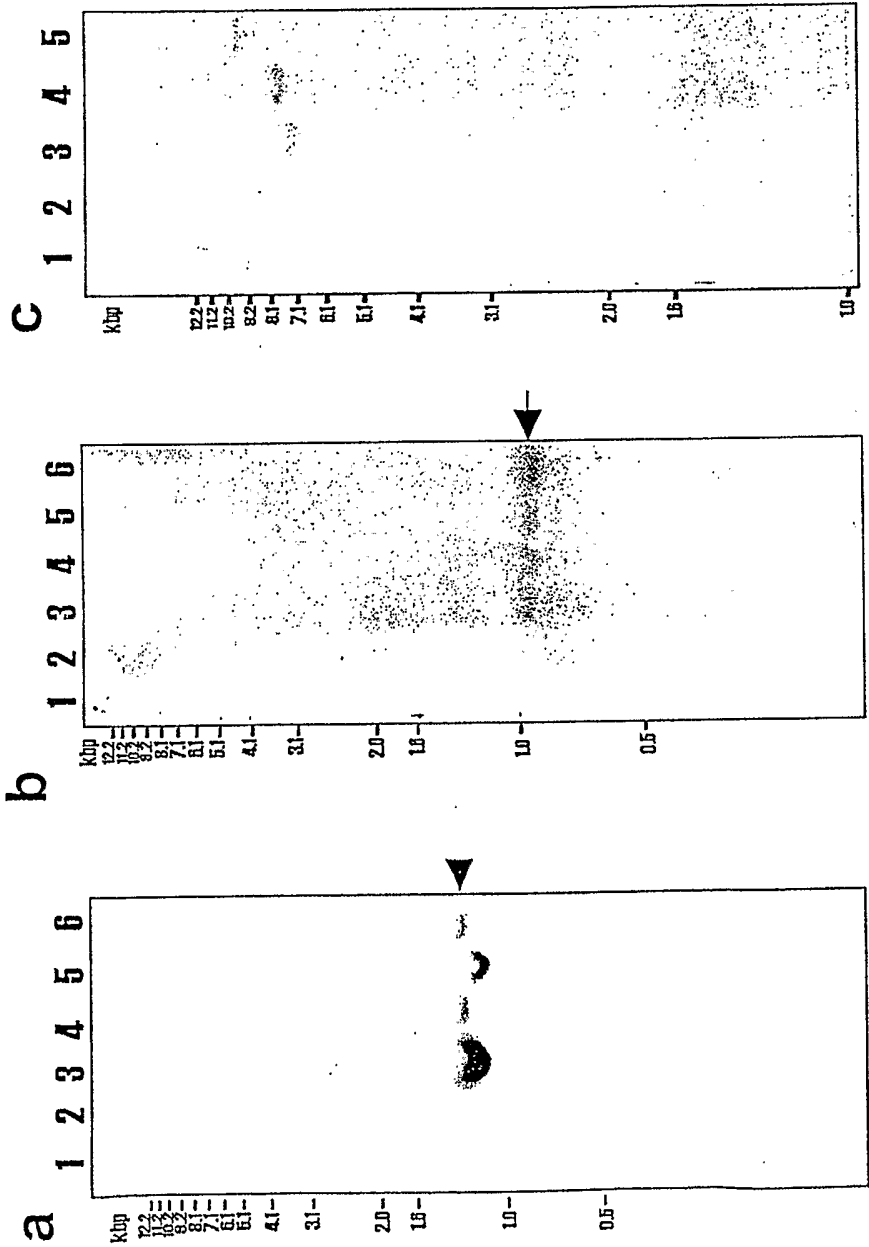


FIG. 10A

FIG. 10B

FIG. 10C

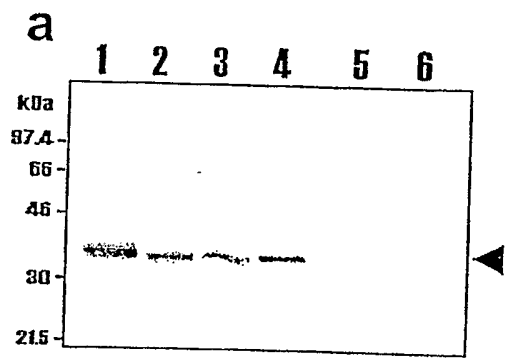


FIG. 11A

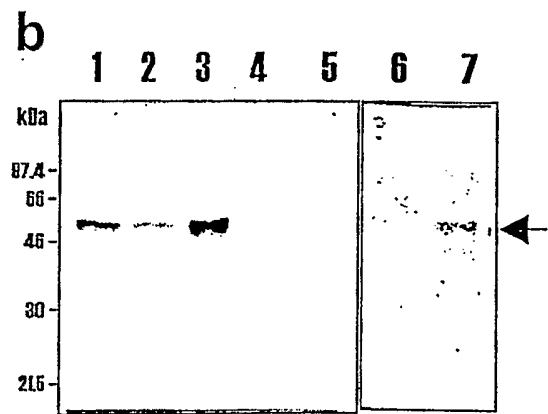


FIG. 11B



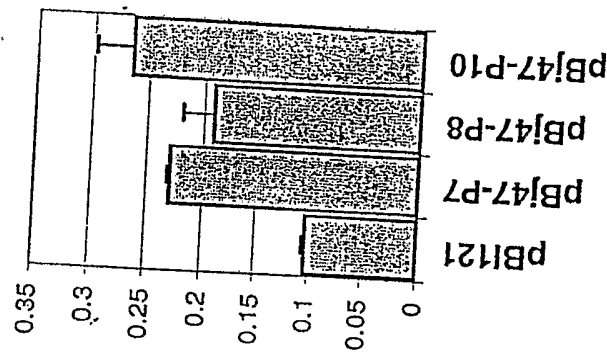


FIG. 12B

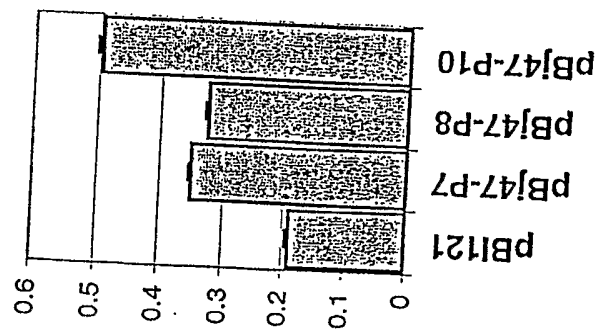


FIG. 12A

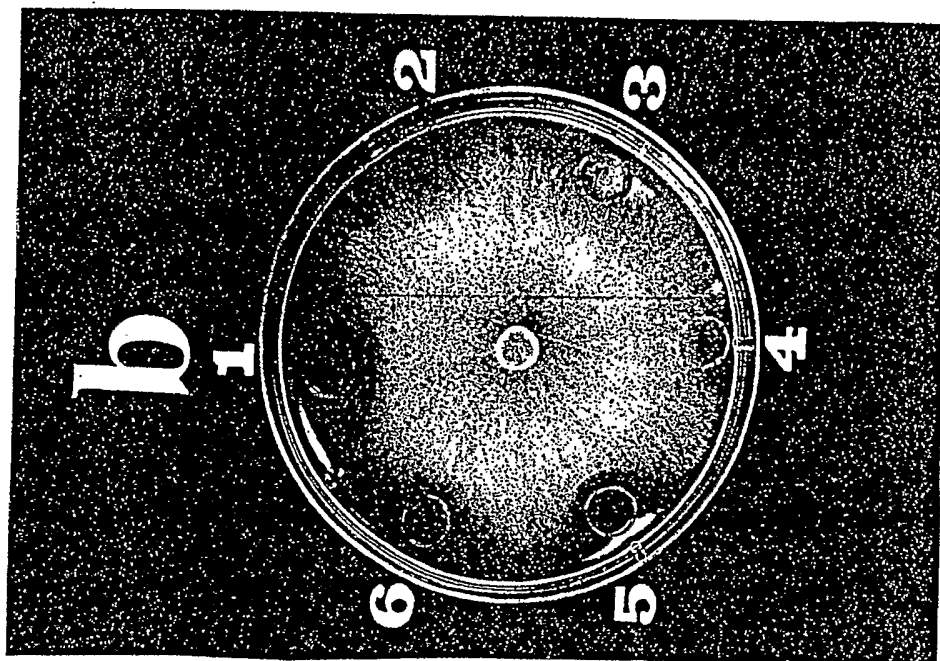


FIG. 13B

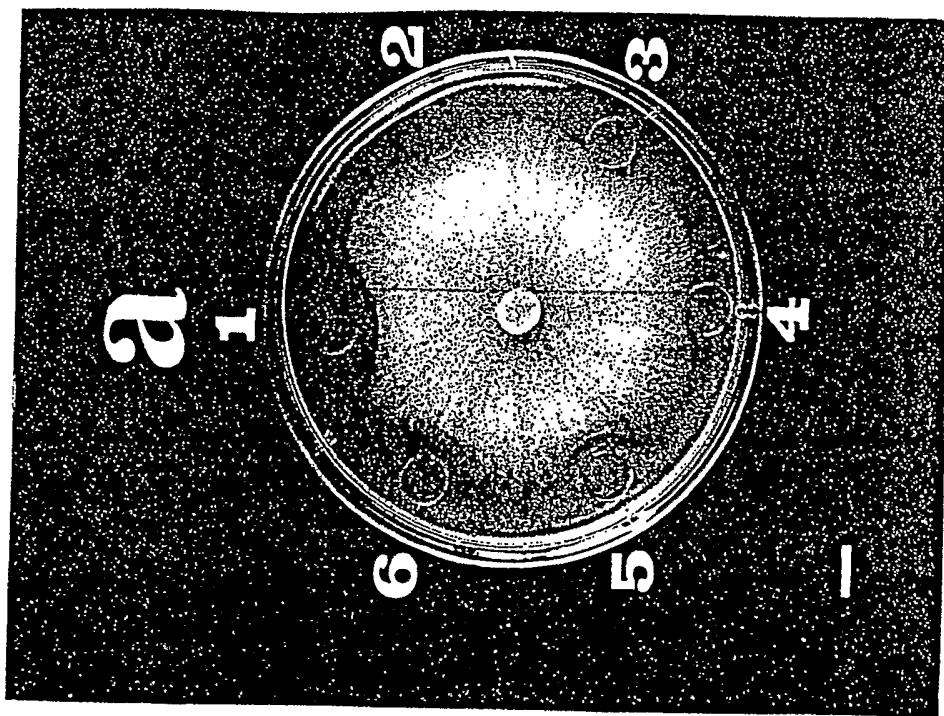


FIG. 13A

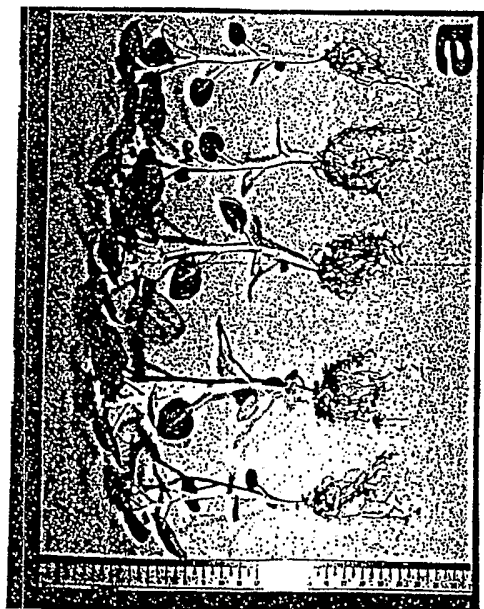


FIG. 14A

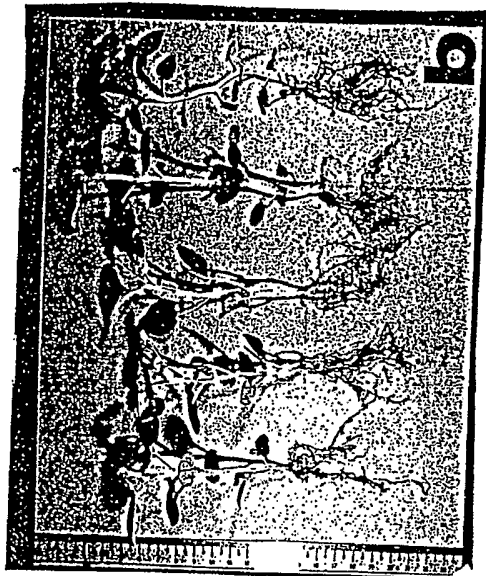


FIG. 14B



FIG. 14C

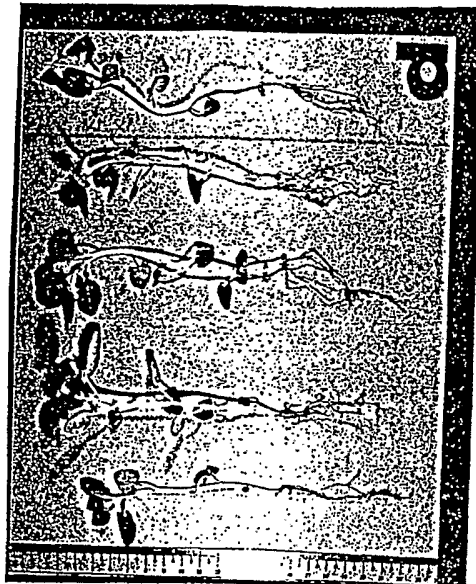


FIG. 14D

## SEQUENCE LISTING

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<120> GENETICALLY MODIFIED PLANTS WITH ENHANCED RESISTANCE TO FUNGAL DISEASES AND A METHOD OF PRODUCTION THEREOF

<130> 9661-025-999

<140>

<141>

<150> 60/331,749

<151> 2001-11-20

<160> 7

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 1297

<212> DNA

<213> B. juncea

<220>

<223> Brassica juncea chitinase (BjCHI1)

<400> 1

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tgcagcgagg ctggatgggt cggcaccacc gaagcttact gcgggcatgg ttgtcaaagc 180
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cccgcgggag ccctctgccc caacgggtcta tgctgtagcg aggctggatg gtgcggcacc 300
accgaagctt actgcgggca tggttgccaa agccagtgc cctccattcc cactcctcct 360
gctcccactc ccactcctcc tactcccact cctcctagtc ctaccctcc tgggtcccact 420
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gcacccgatg gagcaaatac atggggctac tgttacaagg aagaaattga caaaagcgat 720
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cgaggacca tgaatgctgtc ttggaactat aattacggac cgtgcgggag agacctagga 840
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gccatttggt tctggatgac tcctcaagct cctaaaccct cgtgccacga cgtgatcacc 960
gaccagtggg agccgtcggc tgccgacatt tctgccgaa ggttaccagg ttatggagtg 1020
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gatcggatat cgttttatac aaggtactgt ggcattgttg gtgttgatcc tggaaagta 1140
attgactgtg acaatcaaag gccgtttaat gaaggtagta acgttttctt ggatgctgca 1200
atttaataag tactgttaat gaagctttgt tgtatccaag caataagaga gtatcaaatt 1260
aaattaaata aaactccttt ttattaagta aaaaaaa 1297

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<210> 2

<211> 400

<212> PRT

<213> B. juncea

<220>

<223> deduced amino acid sequence of Brassica juncea chitinase (BjCHI1)

<400> 2

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Met Lys Thr Tyr Leu Leu Leu Leu Leu Ile Phe Ser Leu Leu Leu Ser
 1             5             10             15
Phe Ser Ser Gly Glu Gln Cys Gly Ser Gln Ser Ile Pro Glu Gly Ala
          20          25          30
Leu Cys Pro Asn Gly Leu Cys Cys Ser Glu Ala Gly Trp Cys Gly Thr
          35          40          45

```

Thr Glu Ala Tyr Cys Gly His Gly Cys Gln Ser Gln Cys Asn Pro Gly  
 50 55 60  
 Pro Tyr Pro Pro Pro Pro Thr Pro Gln Cys Gly Arg Gln Ser Ile Pro  
 65 70 75 80  
 Ala Gly Ala Leu Cys Pro Asn Gly Leu Cys Cys Ser Glu Ala Gly Trp  
 85 90 95  
 Cys Gly Thr Thr Glu Ala Tyr Cys Gly His Gly Cys Gln Ser Gln Cys  
 100 105 110  
 Thr Pro Ile Pro Thr Pro Pro Ala Pro Thr Pro Thr Pro Pro Thr Pro  
 115 120 125  
 Thr Pro Pro Ser Pro Thr Pro Pro Gly Pro Thr Pro Pro Gly Pro Ser  
 130 135 140  
 Gly Asp Leu Ser Gly Ile Ile Ser Arg Asp Gln Phe Tyr Lys Met Leu  
 145 150 155 160  
 Lys His Met Asn Asp Asn Asp Cys His Ala Val Gly Phe Phe Thr Tyr  
 165 170 175  
 Asp Ala Phe Ile Thr Ala Ala Lys Ser Phe Pro Ser Phe Gly Asn Thr  
 180 185 190  
 Gly Asp Leu Ala Met Arg Lys Lys Glu Ile Ala Ala Phe Phe Gly Gln  
 195 200 205  
 Thr Ser His Glu Thr Thr Gly Gly Trp Ser Gly Ala Pro Asp Gly Ala  
 210 215 220  
 Asn Thr Trp Gly Tyr Cys Tyr Lys Glu Glu Ile Asp Lys Ser Asp Pro  
 225 230 235 240  
 His Cys Asp Ser Asn Asn Leu Glu Trp Pro Cys Ala Pro Gly Lys Phe  
 245 250 255  
 Tyr Tyr Gly Arg Gly Pro Met Met Leu Ser Trp Asn Tyr Asn Tyr Gly  
 260 265 270  
 Pro Cys Gly Arg Asp Leu Gly Leu Glu Leu Leu Lys Asn Pro Asp Val  
 275 280 285  
 Ala Ser Ser Asp Pro Val Ile Ala Phe Lys Thr Ala Ile Trp Phe Trp  
 290 295 300  
 Met Thr Pro Gln Ala Pro Lys Pro Ser Cys His Asp Val Ile Thr Asp  
 305 310 315 320  
 Gln Trp Glu Pro Ser Ala Ala Asp Ile Ser Ala Gly Arg Leu Pro Gly  
 325 330 335  
 Tyr Gly Val Ile Thr Asn Ile Ile Asn Gly Gly Leu Glu Cys Ala Gly  
 340 345 350  
 Arg Asp Val Ala Lys Val Gln Asp Arg Ile Ser Phe Tyr Thr Arg Tyr  
 355 360 365  
 Cys Gly Met Phe Gly Val Asp Pro Gly Ser Asn Ile Asp Cys Asp Asn  
 370 375 380  
 Gln Arg Pro Phe Asn Glu Gly Ser Asn Val Phe Leu Asp Ala Ala Ile  
 385 390 395 400

&lt;210&gt; 3

&lt;211&gt; 1242

&lt;212&gt; DNA

&lt;213&gt; Hevea

&lt;220&gt;

&lt;223&gt; Hevea 1,3-glucanase (HbGLU)

&lt;400&gt; 3

aaattataag caacttttctt ctaattttccc cccttcttaa tggctatctc ctcttcaact 60  
 tcaggaaacta gtagttcctt cccctcaaga actactgtca tgcttcttct gtttttcttt 120  
 gcagcaagcg ttggtataac agatgccag gtaggtgttt gctatggaat gcaaggcaac 180  
 aaccttcac ctgtttcaga ggtcatagct ctctataaaa aatctaacaat cagagaatg 240  
 agaatttatg atccaaatcg agcagtattg gaagccctta gaggtcaaa cattgaactc 300  
 atactagggtg ttccaaactc agatctccaa agccttacca atccttccaa tgcaaaatca 360  
 tgggtacaaa aaaatgttcg tggcttcttg tcaagtgtcc tgttcagata tatagcagtt 420  
 ggcaacgaaa ttagtcctgt caatagaggc acagcttggt tggctcaatt tgttttgct 480  
 gccatgagaa atatacatga tgctataaga tcagctgggtc ttcaagatca aatcaaggctc 540

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tccactgcaa ttgacttgac cctggttaga aattcctacc ctccctctgc aggtgctttc 600
agggatgatg ttagatcata cttggacca attattggat ttctatcctc tatcagggtca 660
cctttacttg ccaatattta tccttacttt acttatgctt ataatccaag ggatattttc 720
cttcctatg ctttggtcac ttcacccatca gttgttggtg gggatgggtca gcgagggtat 780
aagaaccttt ttgatgcaac gttggatgca ttgtactctg ctcttgagag ggctagtgggt 840
ggttctctgg aggtggttgt ttcggaagt ggctggcgt ctgccggagc atttgctgcc 900
acatttgaca atgggcgtac ttatctctca aatttgatcc aacatgttaa aggaggtact 960
cctaagaggc ctaacagagc tatagagact tacttatttg ccatgtttga tgaaaataag 1020
aagcaaccag aggttgagaa acactttgga cttttctttc ctgataaacg gccaaaatat 1080
aatctcaatt ttggtgcaga aaagaactgg gatatttcta ctgaacacaa tgcaacaata 1140
cttttcctta agagtgatat gtgagattgt gagaatttaa gtactatata tatttccaat 1200
gtatgcatgt atccatgtat taaataagag aaccttttct ca 1242

```

&lt;210&gt; 4

&lt;211&gt; 374

&lt;212&gt; PRT

&lt;213&gt; Hevea

&lt;220&gt;

&lt;223&gt; Deduced amino acid sequence of Hevea 1,3-glucanase (HbGLU)

&lt;400&gt; 4

```

Met Ala Ile Ser Ser Ser Thr Ser Gly Thr Ser Ser Ser Phe Pro Ser
1      5      10      15
Arg Thr Thr Val Met Leu Leu Leu Phe Phe Phe Ala Ala Ser Val Gly
20      25      30
Ile Thr Asp Ala Gln Val Gly Val Cys Tyr Gly Met Gln Gly Asn Asn
35      40      45
Leu Pro Pro Val Ser Glu Val Ile Ala Leu Tyr Lys Lys Ser Asn Ile
50      55      60
Thr Arg Met Arg Ile Tyr Asp Pro Asn Arg Ala Val Leu Glu Ala Leu
65      70      75      80
Arg Gly Ser Asn Ile Glu Leu Ile Leu Gly Val Pro Asn Ser Asp Leu
85      90      95
Gln Ser Leu Thr Asn Pro Ser Asn Ala Lys Ser Trp Val Gln Lys Asn
100     105     110
Val Arg Gly Phe Trp Ser Ser Val Leu Phe Arg Tyr Ile Ala Val Gly
115     120     125
Asn Glu Ile Ser Pro Val Asn Arg Gly Thr Ala Trp Leu Ala Gln Phe
130     135     140
Val Leu Pro Ala Met Arg Asn Ile His Asp Ala Ile Arg Ser Ala Gly
145     150     155     160
Leu Gln Asp Gln Ile Lys Val Ser Thr Ala Ile Asp Leu Thr Leu Val
165     170     175
Gly Asn Ser Tyr Pro Pro Ser Ala Gly Ala Phe Arg Asp Asp Val Arg
180     185     190
Ser Tyr Leu Asp Pro Ile Ile Gly Phe Leu Ser Ser Ile Arg Ser Pro
195     200     205
Leu Leu Ala Asn Ile Tyr Pro Tyr Phe Thr Tyr Ala Tyr Asn Pro Arg
210     215     220
Asp Ile Ser Leu Pro Tyr Ala Leu Phe Thr Ser Pro Ser Val Val Val
225     230     235     240
Trp Asp Gly Gln Arg Gly Tyr Lys Asn Leu Phe Asp Ala Thr Leu Asp
245     250     255
Ala Leu Tyr Ser Ala Leu Glu Arg Ala Ser Gly Gly Ser Leu Glu Val
260     265     270
Val Val Ser Glu Ser Gly Trp Pro Ser Ala Gly Ala Phe Ala Ala Thr
275     280     285
Phe Asp Asn Gly Arg Thr Tyr Leu Ser Asn Leu Ile Gln His Val Lys
290     295     300
Gly Gly Thr Pro Lys Arg Pro Asn Arg Ala Ile Glu Thr Tyr Leu Phe
305     310     315     320
Ala Met Phe Asp Glu Asn Lys Lys Gln Pro Glu Val Glu Lys His Phe

```

				325					330					335				
Gly	Leu	Phe	Phe	Pro	Asp	Lys	Arg	Pro	Lys	Tyr	Asn	Leu	Asn	Phe	Gly			
			340						345					350				
Ala	Glu	Lys	Asn	Trp	Asp	Ile	Ser	Thr	Glu	His	Asn	Ala	Thr	Ile	Leu			
		355					360						365					
Phe	Leu	Lys	Ser	Asp	Met													
	370																	

&lt;210&gt; 5

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: C1(forward)primer

&lt;400&gt; 5

ctgaattctc ctccggtgag caatgcg

27

&lt;210&gt; 6

&lt;211&gt; 26

&lt;212&gt; DNA

&lt;213&gt; Artificial

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: C21(forward)primer

&lt;400&gt; 6

ctgaattcgg ggatctttct ggcac

26

&lt;210&gt; 7

&lt;211&gt; 34

&lt;212&gt; DNA

&lt;213&gt; Artificial

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: C2(reverse)primer

&lt;400&gt; 7

gcgactgcgg ccgcgttact accttcatta aacg

34

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CN02/00822

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>7</sup> : C12N15/62,15/56,15/29,15/82,A01H5/00

According to International Patent Classification(IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched(classification system followed by classification symbols)

IPC<sup>7</sup> : C12N15/62,15/56,15/29,15/82,A01H5/00

Documentation searched other than minimum documentation to the extent that such documents are included in the field searched

Electronic data base consulted during the international search(name of data base and, where practicable, search terms used)

WPI,GenBank+EMBL+DDBJ+Swiss-Prot+PIR+PDB,EPODOC,CNPAT,PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant claim No.
A	US6087560 A, (MOGEN INT NV., RIJKSUNIV LEIDEN) ; A01H5/00; 2000/07/11. See the abstract.	1-19
A	CN1229141 A, (BIOTECH RES CENT CHINESE ACAD AGRI) ; A01H5/00; 1999/09/22 See the abstract.	1-19
A	US5670706 A, ( MOGEN INT NV., RIJKSUNIV LEIDEN) ; C12N15/82; 1997/09/23. See the abstract.	1-19
A	DE4234131 A1, (PLAC, et al) ; A01H5/00; 1994/04/21 See the abstract.	1-19
A	EPO440304 A, ( MOGEN INT NV., et al) ; A01H5/00; 1991/08/07. See the abstract.	1-19



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“E” earlier document but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason(as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&amp;” document member of the same patent family

Date of the actual completion of the international search

24 March 2003 (24.03. 03)

Date of mailing of the international search report

10 APR 2003 (10.04.03)

Name and mailing address of the ISA/

The Chinese Patent Office  
6, Xitucheng Road, Haidian District,  
Beijing, 100088, China

Facsimile No. 86-010-62019451

Authorized officer

ZENG, Fanhui

Telephone No. 62093733



**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.

PCT/CN02/00822

Patent document cited in search report	Publication date	Patent family members	Publication date
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		EP0616035 A2	1994/09/21
		US5689045 A	1997/11/18
		US5804184 A	1998/09/08
		US6271438 B1	2001/08/07
EP0440304 A	1991/08/07	AU7003491 A	1991/08/01
		CA2035134 A	1991/07/31
		JP8280283 A	1996/10/29
		US6066491 A	2000/05/23
		DE69132479 E	2001/01/04